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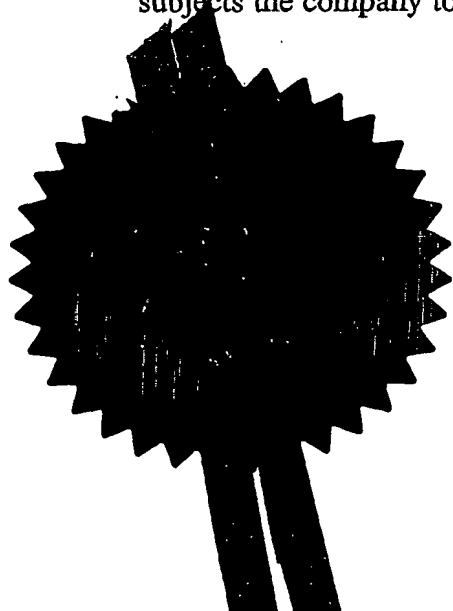
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*R. Mahoney*

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Dated 18 March 2003

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1. Your reference 59.75763

2. Patent application number  
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3. Full name, address and postcode of the  
or of each applicant (underline all surnames)  
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8310179001

Patents ADP number (if you know it)

If the applicant is a corporate body, give  
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4. Title of the invention A Method

5. Name of your agent (if you have one) Frank B. Dehn & Co.

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11.

We request the grant of a patent on the basis of this application

Signature Rebecca Gardner

Date 23 January 2002

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75763.358

A Method

5       The present invention relates to methods of screening molecular libraries and to the uses of molecules identified by such screening methods.

10       Gene therapy has yet to establish itself as the technique of choice for treatment of genetic diseases and of cancers caused by genetic mutations. Despite the new wealth of genetic information, the initial enthusiasm about the potential for gene therapy has been dampened by practical problems encountered in providing on effective therapy for most diseases having a genetic

15       cause. The basis principle of such gene therapy is that a nucleic acid construct which encodes a target protein in its wild type form can be introduced into a patient's cells, to counteract deficiencies in expression or mutations in the version of the target protein normally

20       expressed by the patient. Thus, a patient receiving gene therapy will typically produce the recombinant wild type protein in addition to the defective protein encoded by their own genome.

25       A large number of genetic defects/diseases are known but from these only a limited gene-list for gene therapy candidates exists. Typically, in order to encode a whole protein, the corresponding gene/cDNA must be generated and introduced into the patient in order to express the encoded protein under the regulation of a

30       suitable promoter. Large DNA sequences pose a very high risk of containing immunogenic sequences that can lead to adverse effects, especially with repeated administration of the gene/vector combinations. In addition, while the use of viral vectors for treating

35       humans is undesirable, most current gene therapy trials have relied on viral-based vectors.

There is therefore a need for an alternative to

gene therapy for treating genetic disorders, preferably a method which relies on the administration of a relatively simple molecule. Pharmaceutical companies and the medical profession have much expertise in the  
5 administration and production of small organic compounds, peptides, steroids etc. and therefore a range of suitable carriers and methods of administration are available once a compound is found to be effective.

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~~Protein therapy has been proposed which involves~~

10 the introduction of a replacement wild type protein but again, the administration of a whole protein, while possible with small proteins like insulin, is much more difficult when the deficient or mutant protein is large.

It has long been assumed that the primary structure  
15 of a protein, i.e. the amino acid sequence (which is in turn dependent on the nucleic acid coding sequence), determines the tertiary/quaternary structure of the protein (see, e.g. Anfinsen, C. (1986) Protein Engineering Ed. Inouye and Sarma pp 3-13). However, we  
20 have established a method that does not rely on replacement of the mutated gene, but rather aims at the correction of the protein expressed by the mutated gene. Thus, while the mutant protein may have an altered three-dimensional structure due to a non-wild type  
25 primary structure, the three-dimensional structure can be altered without correcting the primary structure. Preferably, small molecules, e.g. peptides or small organic molecules, may be used which interact with the mutant protein to modify its three dimensional structure  
30 and thus its activity. Proteins are known to change their conformation on binding or associating with a range of different classes of molecules, e.g. other proteins, nucleic acids, prosthetic groups such as heme, ions such as  $\text{Ca}^{2+}$  etc.

35 It is now proposed to utilise small ligands to alter the three-dimensional structure of a mutant protein to restore or alter the function or activity of

that mutant protein. Typically the ligand can restore, in whole or in part, wild type protein function. It could perhaps be attempted to design molecules, e.g. through computer modelling, which it is hoped would have the desired impact on the target mutant protein. However even with sophisticated modelling programs, it is still difficult to build in accurately all the possible conformational changes that could occur on ligand binding, particularly with a complicated protein. Moreover, it would still be necessary to test these candidate molecules for their activity in vitro/in vivo.

A solution to these problems is offered by the present invention, which according to one aspect provides a method of screening a library of molecules for the ability of members of that library to restore or modify the function of a target protein in an intracellular environment, which method comprises introducing the library into cells which have a reporter system which allows the identification of those cells in which the function of the target protein has been restored or modified.

The library of molecules under investigation will typically be peptide molecules or other organic molecules, whether organically synthesised or natural products. Such libraries of organic compounds are now commercially available. Peptidomimetics which may have a peptide like backbone and functional groups appended thereto are a useful class of small organic molecules.

The term "peptide" is used herein to encompass molecules which might be termed "oligopeptides" or "polypeptides" by those in the art. It will be appreciated that where the members of the library having the ability to restore or modify protein functions are peptides, the peptide library may actually be introduced into the cells in the form of a library of nucleic acid molecules or constructs (preferably DNA plasmid vectors) which encode the peptide molecules to be screened and

investigated. This is, in fact, a particularly preferred way of performing the invention and is discussed in more detail below and in the Examples. Alternatively, a library of peptide molecules may be  
5 introduced directly as peptides, which have been expressed outside the cells having the reported system or chemically synthesised.

It has surprisingly been found that very small peptides can restore or modify the function of target  
10 proteins through interacting with them *in vivo* and causing changes in their conformation. The members of a peptide library therefore typically have no more than 12 amino acids, preferably 2-8 amino acids. When a library of non-peptides molecules is analysed, they will  
15 generally be of comparable or even smaller size.

Because of the suitability of peptide/peptide interactions in promoting conformational changes, peptidomimetics are a preferred class of non-peptide molecules which may make up the library screened in  
20 accordance with a method of the invention. The design and production of peptidomimetics is now well known in the art and the skilled man is aware of how such molecules may be prepared and screened. Many pharmaceutical and other companies have their own banks  
25 of peptidomimetics and other organic compounds which could be used in the *in vivo* screening methods of the present invention. The following website provides details of a number of suppliers of compound libraries, such as Advanced ChemTech, Affymax, BioLeads GmbH, ComGenex, Inc. etc.,  
30 <http://www.htscreening.net/suppliers/comblib.html>.

We describe a method of "screening a library". While the library may have been selected or designed to have certain structural motifs and the nature of  
35 individual members of that library can be assumed, this phrase implies that it is not known which member is introduced into which cell (or cell sample) and the

molecules are not introduced in a controlled series.

5 The library which is screened in accordance with the present invention will typically have at least 96 different members, which are usually screened at the same or substantially the same time, e.g. the library members are in contact with the host cells at the same time or at least screened as part of a series of coordinated multiplex assays. Preferably the library will comprise at least 500 different members (species)  
10 more preferably at least 1000, e.g. at least 5000, particularly preferably at least 10,000 or even at least 25,000 members.

15 The library may be screened all together or in batches but in that case at least 8, preferably at least 64 or 96 different library members would still be contacted with the host cell population at the same time.

20 A suitable protocol for screening chemical libraries would be to use cancer cells containing an engineered reporter/screening system. These cells are placed with a culture medium on microtitre plates, where one compound from the library is present in each well, thus non-peptide libraries may be screened in batches.

25 Non-peptide libraries or peptide libraries which are not encoded by mini-genes may be screened on an array. Chemical arrays having 500 eg 1,000 or more different compounds with each compound identified to a fixed address within that array are commercially available. The screening methods of the present  
30 invention are particularly effective in this regard as positive results are only obtained with molecules which can enter the cell, restore or modify target protein function and are non-toxic.

35 The method provided is a "screening method" in that performance of the method gives information about individual members of the library of molecules. The method is performed in an intra-cellular environment

(i.e. *in vivo*), and therefore all toxic compounds will be weeded out very early on. This reduces the cost of screening and of pre-clinical and even phase I clinical trials. The method is able to indicate in a qualitative  
5 possibly also quantitative manner the performance of individual molecules in the test system. A target protein and suitable host cells are selected and the method designed so that the reporter system is able to give information about the ability of each molecule to  
10 restore or modify the function of the target protein. In preferred embodiments, the ability of members of the library to restore the function of the target protein is assessed although alternatively it may be useful to investigate the potential for modifying protein  
15 function.

Thus, when the function of the target protein has been "restored" a form of protein therapy has taken place which advantageously relies on administration of small modulating molecules rather than a full  
20 replacement protein (or a nucleic acid molecule encoding for the full protein). Restoration of function is determined in relation to the function, in a physiologically relevant respect, of the wild-type protein found in healthy cells. Mutant variants of  
25 proteins may exist in cells and contribute to various disease conditions, for example many cancers are caused by the presence in certain cells of the body of one more mutant versions of naturally occurring proteins, e.g. proteins responsible for cell growth and the normal  
30 working of the cell cycle. Numerous other diseases are caused by the presence in cells of a mutant version of a naturally occurring protein, e.g. cystic fibrosis and sickle cell anaemia, phenylketonuria, multiple carboxylase deficiency, methylpurine DNA glycosylase  
35 deficiency (MPG), ataxia, chemotherapy resistance due to mutations in the gene coding for methylguanine - DNA methyl transferase (MGMT) etc.

The reporter system is typically designed to measure restoration of a particular function of the target protein, for example the ability to act as a DNA binding transcription factor. Thus restoration of function refers to a return to wild-type function of the protein in at least one physiologically relevant and measurable respect. In many cases, the wild-type functions of the target protein in all respects may be restored but this is not essential for the successful working of the invention. It will be appreciated that partial restoration of the function of a protein may still be useful and it is not a requirement for a positive identification according to the claimed screening method to provide return to 100% of wild-type activity.

The sensitivity of the reporter system may conveniently be modified to provide a more or less stringent assay and thus to identify as giving "positive" results only those molecules which have achieved a significant increase in protein function. Preferably, molecules giving positive results according to the claimed screening methods will have brought about at least a 30%, more preferably at least a 50%, particularly preferably at least a 70% restoration in wild-type protein function, with respect to the particular function monitored by the intra-cellular reporter system. In some instances, a ligand may correct the mutant to a level of measured activity which is actually better than wild type. It will be clear to the skilled reader that the art provides a number of ways of comparing the relative activities of different molecules. Some reporter systems e.g. those based on fluorescence may be quantitative and thus facilitate such a comparison between wild-type and corrected mutant. However an assessment relative to the wild-type protein may more conveniently be performed as part of a separate test utilising serial dilutions, measurement of

plaque size etc.

Unless otherwise clear from the context, the above discussion also applies to the circumstance where a particular function/activity of a protein is modified.

5 "Modification" of function refers to the situation where the function of a wild type protein in one or more physiologically relevant respects is altered, e.g. to enhance or decrease binding affinity for an enzyme's co-factor or substrate, or ability to promote gene  
10 expression through DNA binding.

Suitable target proteins for restoration of function will include those whose presence in mutated form is associated with a disease state. The mutated version of the protein will typically have an altered 3-  
15 dimensional structure which affects its ability to interact with other molecules (ions, intra-cellular organelles and other cell components etc.). The members of the molecular library will be screened for their ability to interact with the mutant target and thus  
20 alter 3-dimensional structure. The 3-dimensional structure *in vivo* may be closer in one or more respects to the wild-type 3-dimensional structure as a result of successful interaction with a member of the library but protein function may be restored through a further  
25 compensating change in 3-dimensional structure which has a return to wild-type function as a 'net' result. Preferred target proteins will be those wherein point mutation(s) are the cause of disease. Particularly preferred are transcription factors (DNA binding  
30 proteins) such as p53, but others would be enzymes, peptide hormones or receptor molecules. All diseases caused by monogenic Mendelian mutations could be targets for treatment with molecules identified according to the screening methods of the present  
35 invention. These include genetic (i.e. hereditary) diseases, cancer and symptoms of aging caused by mutations. Specific diseases such as MPG, MGMT etc. are

listed above.

Key to the presently claimed screening methods is the fact that the library is screened *in vivo*, i.e. the influence of each molecule within an intra-cellular environment is monitored. This offers considerable advantage over *in vitro* methods of screening lead compounds such as those using phage display, for example, which relies on binding/screening taking place outside the phage. Phage display is further limited in that putative binding ligands are expressed as an integral part of a phage surface protein. In a preferred embodiment of the present invention, the library of molecules is introduced into the host cells in the form of plasmids which express the peptides to be screened freely, without the need to conjugate with an existing cellular protein.

Suitable host cells are chosen which are capable of expressing the target protein. Preferably the host cells are eukaryotic, more particular of human origin, although it may be desired to manipulate a prokaryotic, e.g. bacterial protein in which case prokaryotic host cells will be used. The host cells should provide a suitable model for determining whether the members of the library are capable of causing the desired change in target protein function with a view, typically, to therapeutic administration of successful members of the library or a derivative thereof. Thus, where protein function restoration is required, the host cells will typically be derived from cancerous or other diseased cells and naturally produce the dysfunctional target protein. Suitable cell lines are available, for example in cell culture collections such as the ATCC and may be derived from osteosarcoma, adenocarcinoma etc., or indeed any established cell line that expresses the mutant protein. Cell lines that lack any expression of the protein of interest can also be used after introducing and expressing the gene encoding the mutated

protein. Similarly, cells from other organisms that lack the corresponding gene that can complement the function of such a cell can also be used after introducing and expressing the gene encoding the mutated  
5 protein. Likewise, when the ability of members of a library to modify protein function is under investigation, host cells will typically be chosen which naturally express the target protein under normal growing conditions.

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10           Methods for introducing the library of molecules into host cells are well known in the art. In the preferred embodiments wherein the library is introduced in the form of nucleic acid molecules encoding peptides of interest, standard transfection techniques may be  
15 used. Where the library to be introduced comprises peptides or organic molecules, the members of the library may suitably be introduced into host cells by adding members of the compound library to the culture media eg. in the individual wells of a microtitre plate  
20 or through the use of microtites, plates or chips on which members of the chemical library are arrayed.

          The appropriate reporter system for a given screening method will depend upon the nature of the target protein under investigation. The reporter system  
25 is chosen to be responsive to the target protein and thus be capable of indicating the functional status of the target protein. The mammalian p53 protein illustrates the principle behind a suitable reporter system. P53 functions in mammalian cells mainly through  
30 the ability to act as a transcription factor and is a transcriptional activator of several genes associated with cell cycle regulation such as p21, Bax, CD95(Fas/Apo-1) and 4-3-3 $\sigma$  (HME1). It can also down regulate the transcription of cell-cycle-regulating  
35 genes such as Bcl2, Cdc2 and cyclin. P53 acts as a checkpoint, monitoring DNA damage and regulating cell cycle progression. Loss of p53 activity predisposes

cells to the acquisition of oncogenic mutations and may  
favour genetic instability - 90% of mutations reported  
in the p53 mutation database are found in the DNA  
binding domain. Mutations in p53 can lead to cancer  
5 formation where p53-mediated apoptosis is deficient due  
to the mutation.

P21 is one of the p53-transactivated genes that are  
critical in cell cycle control and many p53 mutants  
found in cancer cells lose the ability to transactivate  
10 p21 transcription. Thus the transactivation level of  
p21 promoter in a cell reflects whether the p53 protein  
expressed in that cell has wild type function. As  
discussed in more detail in the Examples, a p53 reporter  
system was constructed by cloning human p21 promoter  
15 upstream of the puromycin resistance gene. The reporter  
construct can provide human cells with puromycin  
resistance only in the presence of wild type or wild-  
type functioning p53 that transactivates the p21  
promoter. Such a reporter system offers significant  
20 practical benefits as a life-death selection system is  
possible where only cells with wild-type-functioning p53  
can survive when grown in the presence of puromycin.

Thus, while the host cells may have a reporter  
system as part of their normal genome which can be  
25 utilised, typically the host cells will have been  
modified to include a suitable gene based reporter  
system. The genetic constructs which comprise all or  
part of the reporter system will thus have been  
introduced into the host cells, e.g. by standard  
30 transformation/transfection techniques, before the  
screening method is performed. In certain  
circumstances, the reporter constructs could be  
introduced at the same time as the molecular library or  
even, but not preferably, after introduction of the  
35 library. Thus for performance of the screening methods  
of the invention, host cells will preferably have been  
co-transfected (although not necessarily simultaneously)

with a reporter construct and a construct which encodes a peptide, or by introducing members of a chemical compound library, whose ability to restore or modify the function of a target protein is to be investigated.

5       As described in the following Example relating to restoration of function of a mutant form of the *E.coli* tryptophan repressor, two reporter systems, i.e. two different reporter constructs may be used e.g. with one acting as a positive selection survival system and one  
10      to confirm restoration potential of the screened peptides.

Target proteins are preferably DNA binding proteins which up or down regulate the expression of other genes through binding to promoter or enhancer regions. Their  
15      native gene targets may be utilised to report on whether a target protein function has been restored or modified, but preferably their target DNA binding regions will be operably linked to reporter genes such as genes  
20      fluorescent proteins such as GFP (green fluorescent protein), or the much used bacterial reporter enzymes  $\beta$ -galactosidase ( $\beta$ -Gal) or  $\beta$ -glucuronidase ( $\beta$ -Gus) etc. According to a particularly convenient and preferred method the protein products of these reporter genes  
25      (e.g.  $\beta$ -Gal or  $\beta$ -Gus) could be fused to a signal peptide in order to 'display' the proteins on the cell surface. In this way, the cells expressing the reporter protein can readily be separated physically from other cells that do not express the reporter protein, e.g. by the  
30      use of antibodies. Suitable signal peptides are described in the literature and include the signals from a protein such as PDGFR (platelet-derived growth factor receptor). A typical construct would thus be as follows:

35      Secretion signal peptide - reporter protein (eg.  $\beta$ -Gal/ $\beta$ -Gus) - transmembrane domain  
METDTLLLWVLLLWVPGSTGD - reporter protein (eg.  $\beta$ -

Gal/ $\beta$ -Gus) - AVGQDTQEVIVVPHSL  
PFKVVVISAILALVVLTIISLIILIMLWQKKPR - stop.

5 Non-DNA binding proteins can also be used as target proteins provided their wild type activities can be monitored and assayed for or the reporter system engineered such that cell survival depends on restoration of the protein function.

10 Identification of cells in which the function of the target protein has been restored or modified(e.g. wild-type function has been restored) will depend on the reporter system used. As described above, this is particularly conveniently performed when cells in which wild-type function has not been restored do not survive in the selected culturing environment. Fluorescence  
15 detection can also be used to identify cells in which the function of the target protein has been restored or modified. The target protein may, for example, be an enzyme rather than a nucleic acid binding protein and the ability of the target enzyme to act on a substrate  
20 may be used as the intra-cellular reporter system allowing identification of cells in which the function of the target protein has been restored or modified. Other suitable reporter system such as the  $\beta$ -gal. blue/white system are well known in the art and can be  
25 used or adapted by the skilled man in his chosen screening method.

In a further aspect, the present invention relates to molecules identified by the screening methods described herein or to derivatives of these molecules.  
30 Methods of treatment utilising such molecules and their use in therapy constitute still further aspects of the invention.

Through application of a screening method as described hereby it has surprisingly been found that  
35 small molecules e.g. peptides of less than 10 amino acids in length are capable of restoring wild-type function of p53 protein. It is thought that at least

60% of cancers are due to a mutation in p53 which affects function. Ligands identified by the screening methods of the invention have been shown to restore p53 function and cause death of cancer cells.

5        Thus, in a further aspect, the present invention provides peptides of 2 to 10 amino acids in length, or derivatives thereof, which restore wild-type function of human p53 for use in therapy. Alternatively viewed, the present invention provides peptides of 2 to 10 amino

10       acids in length, or derivatives thereof for use in restoring restore wild-type function of human p53.

      The present invention also provides the use of peptides of 2 to 10 amino acids in length, or derivatives thereof, which restore wild-type function of  
15       human p53 in the manufacture of a medicament for treating cancer.

      Similarly, the invention provides a method of treating cancer in a patient which method comprises administration of one or more peptides of 2 to 10 amino  
20       acids in length, or derivatives thereof, which restore wild-type function of human p53.

      Peptide "derivatives" as referred to herein include peptides which incorporate side chain modification(s) e.g. modifications of the N-terminal amino group (e.g. by acetylation), the carboxy-terminal group (e.g. by  
25       amidation or reduction) or one or more amino acid side chains (e.g. by phosphorylation, acetylation, hydroxylation etc.). Modifications will typically limit degradation of the peptide *in vivo* and D-amino acids may  
30       also be incorporated to reduce degradation.

      "Derivatives" also include pharmaceutically acceptable salts which have the ability to restore the function of p53 and do not produce any undesirable toxic effects. Examples of suitable salts are the addition salts of  
35       inorganic or organic acids such as hydrochloric, phosphoric or acetic acid.

      The methods of the present invention are useful for

identifying a candidate molecule which can be used as the starting point for development and optimisation work to identify a potential therapeutic molecule. According to the normal techniques of drug development, site  
5 directed mutagenesis or site saturation mutagenesis may be used to modify a promising molecule identified by the screening methods of the present invention. Thus in a further embodiment, the present invention provides the use of a molecule identified by a screening method as  
10 described herein in a design process to manufacture a therapeutic compound. Such a design process may involve targetted and random modifications of the starting molecule, the generation of degradation resistant variants thereof or non-peptide mimetics thereof as well  
15 as *in vivo* or *in vitro* methods of testing these molecules. The invention also relates in an alternative aspect to the use in therapy (e.g. in restoring the function of a target mutant protein) of the molecules resulting from this design process.

20 The Examples describe suitable methods for determining whether a given peptide or peptide derivative can restore p53 function. As described hereinbefore a useful improvement in p53 function may be achieved without a return to 100% of wild type activity  
25 and references to peptides "which restore wild-type function" must be interpreted with that in mind.

Preferably the peptides are 3 to 7 amino acids in length, e.g. 3 to 5 amino acids in length. Particularly preferred peptides are described herein and include  
30 pentamers whose first amino acid is Met and whose second amino acid is Gly Met or Val, the remaining 3 amino acids are preferably, Trp, Cys and Thr (in that order). Further particularly preferred peptides will be based on the peptides identified according to the screening  
35 method described herein but lack the first Met and second Met, Gly or Val residues as these are required for (stable) expression from a mini-gene but may not be

necessary (or even desirable) when the peptide or derivative of it is administered. In order to keep the molecules as small as possible, the peptide or mimetic thereof will preferably have no more than 4 amino acids.

5        These small peptides and peptides derivatives are therapeutically convenient active agents and can be readily formulated for administration and delivery.

         The peptides, in the form of pharmaceutical compositions, may be administered orally or parenterally  
10    by the subcutaneous, intramuscular or intravenous route. Due to their small size, the peptides may be administered by nasal inhalation. The compositions comprise one or more peptides together with a  
15    pharmaceutically acceptable carrier therefor and optionally, other therapeutic ingredients. The total amount of active ingredients in the compositions varies from 99.99 to 0.01 percent of weight. The carrier must be acceptable in the sense that it is compatible with  
20    other components of the composition and is not deleterious to the recipient thereof.

         The compositions may be formulated according to techniques and procedures well known in the art and widely described in the literature, and may comprise any of the known carriers diluents or excipients. Thus, for  
25    example, compositions of this invention suitable for parenteral administration conveniently comprise sterile aqueous solutions and/or suspensions of the pharmaceutically active ingredients preferably made isotonic with the blood of the recipient, generally  
30    using sodium chloride, glycerin, glucose, mannitol, sorbitol, and the like. In addition, the compositions may contain any of a number of adjuvants such as buffers, preservatives, dispersing agents, agents that  
35    promote rapid onset of action or prolonged duration of action and the like.

         In addition, the present invention relates to non-peptide compounds showing the same restorative effect on

p53 function as displayed by their peptide counterparts. Such peptidomimetics capable of mimicking the activity of the naturally occurring peptide are likely to be better suited for e.g. oral delivery due to their increased chemical stability.

It is now commonplace in the art to replace peptide or protein-based active agents e.g. therapeutic peptides with such peptidomimetics having functionally-equivalent activity. Various molecule libraries and combinatorial chemistry techniques exist and are available to facilitate the identification, selection and/or synthesis of such compounds using standard techniques. The screening methods of the present invention can of course be used to identify suitable peptidomimetics which have the desired restorative effect on p53 function, or a peptide may be identified by the screening method and a peptidomimetic equivalent generated. The Examples herein contain methods which can be used to verify the activity of a candidate molecule in restoring protein function.

The invention will now be described in more detail in the following non-limiting Examples which show, *inter alia*, how molecules may be screened for their ability to affect the function of a target protein *in vivo*, with reference to the figures in which:

Figure 1 provides a schematic representation of the construction of p53 reporter p21<sub>luc</sub> that works in mammalian cells. The p53 transactivated p21 promoter was cloned upstream of puromycin resistant gene, which provided the puromycin resistance to mammalian cells once it was transfected into the mammalian cells which express wild-type functioning p53.

Figure 2 provides a schematic representation of the generated peptide library plasmid. Each peptide in the library is composed of three random amino acids after

two N-terminal amino acids Methionine (Met, or M) and Glycine (Gly, or G). The next three 'X' stands for any amino acid that forms a library consisting of randomly distributed three-amino-acid peptides. The peptide-coding DNA-fragment was inserted into the mammalian expression vector pCEP4 between its KpnI and HindIII site under the CMV promoter. The plasmid has OriP that enables the replication of the plasmid inside mammalian cells. ~~Selection marker expression cassette in the~~ plasmid provides the plasmid-transfected cells with hygromycin resistance.

Figure 3 gives the results of DNA sequencing of the selected ligand (b) and the empty vector pCEP4 (a), both showed the region between KpnI and HindIII. Both were sequenced by EBV Reverse primer, thus resolved in anti-sense chain. The amino acid sequence of the selected ligand was shown under its encoded anti-sense DNA sequence.

Figure 4 gives the results, in the form of gel photographs, of RT-PCR analysis of mRNA level of p53 transactivated gene in SW480.7 cells. Results showed that mRNA levels of wild-type p53 transactivated genes p21/WAF1, 14-3-3 $\sigma$  and CD95 were much higher in SW480.7 cells transfected with ligand than that with empty pCEP4. The constitutive expression gene  $\beta$ -actin which was used to normalize total mRNA amount between samples. Arrows point out bands for the target genes.

A. RT-PCR for p21/WAF1; B. RT-PCR for 14-3-3 $\sigma$ ; C. RT-PCR for CD95; D. RT-PCR for  $\beta$ -actin.

Figure 5 provides a schematic representation of the construction of pBaxur used in the reporter system described in Example 5.

Figure 6 provides a schematic representation of the construction of pIRES-EGFP, used in the reporter system described in Example 5.

5     Figure 7 provides a schematic representation of the construction of pBaxurEGFP or p21urEGFP, used in the reporter system described in Example 5.

10    Figure 8 provides a schematic representation of the construction of pBaxurEGFP-hisD.

15    Figure 9 shows in diagrammatic form the strategy to screen active ligands that restore the function of cancer causing mutated p53. Here Microgenex (a library of mini-genes encoding peptide ligands) are used but the same strategy can be modified to screen non-peptide ligands.

20    Figure 10 is a cartoon giving an overview of the dual selection system of Example 6. In A, the expression the mutant phenylalanyl-tRNA synthase (PheS) permits the incorporation of D,L-p-Cl-Phenyl-Alanine into the growing polypeptide chain resulting in cell death. In B, the restored TrpRT44M mutant binds to the operator promoter fragment and blocks transcription of mutant PheS allowing colonies to form. Likewise, in C the transcription of LacZ is blocked resulting in white colored colonies. Failure of restored TrpRT44M to block LacZ transcription results in blue colonies, seen in D.

25    The pPepLib plasmid is isolated from surviving white colonies and re-transformed into fresh selective conditions to ensure that the TrpR restoration was not transient.

30

Example 1. Construction of p53 reporter working in  
mammalian cells

5 The dysfunction of mutant p53 in cancer cells can result  
from not only the inability in binding to its specific  
recognized consensus DNA sequence, but also the mal-  
localization inside cells or incompetent to interact  
correctly with other transcription co-factors

---

10 (Vogelstein et al., 2000; Xu & El-Gewely, 2001). The  
sub-cellular localization mechanism and transcription  
machinery in mammalian cells is different from that in  
prokaryotic organisms. The loss of specific DNA binding  
ability may engage the post-translational modification  
15 processes that are totally different from that in  
prokaryotic organisms. Thus a p53 reporter for mammalian  
cells has more advantages than that for prokaryotic  
cells.

20 P53 protein functions in mammalian cells mainly through  
its transcriptional factor activity. P21 is one of the  
p53-transactivated genes that are critical in cell cycle  
control. P53 can induce cell cycle arrest in G1 phase  
via transactivation of p21 gene once the cells are under  
stresses such as DNA damaging. Lots of p53 mutants found  
25 in cancer cells lose the ability to transactivate p21  
transcription. This leads to not only the vast  
development of cancer, but also the resistance to cancer  
chemotherapy and radiotherapy that aim to damage DNA in  
order to kill cancer cells (El-Diery et al., 1993). Thus  
30 the transactivation level of p21 promoter in a cell  
reflects whether the p53 protein expressed in that cell  
has wild type function or not.

35 We constructed our p53 reporter by cloning human p21  
promoter upstream of the puromycin resistant gene. It  
has been reported that basal transcription level from

p21 promoter is very low. The reporter can provide human cells puromycin resistance only in the presence of wild type or wild-type-functioning p53 protein that transactivates p21 promoter, while leaving the cells sensitive to puromycin with dysfunctional mutated p53 protein. The advantage to use puromycin resistant gene is to set up a life-death selection: only the cells with wild-type-functioning p53 protein can survive from puromycin selection.

## Materials and Methods

Plasmid and vectors used in this Example are WWP-Luc (gift from Prof. Stanbridge E.J.), pPUR (Clontech), and pUC18 (Pharmacia). Enzymes used for cloning are EcoRI (Promega), PvuII (Promega), SalI (Promega), SphI (Promega), T4 DNA ligase (New England BioLab), Klenow (Promega), and SAP (Shrimp Alkaline Phosphatase). Cell lines for the validation of our reporter are U-2 OS cell (ATCC Number: HTB-96, Homo sapiens (human), osteosarcoma, bone, wild type p53), Saos-2 cell (ATCC Number: HTB-85, Homo sapiens (human), osteosarcoma, bone, p53 null), and SW480.7 (ATCC number: CCL 228, Homo sapiens (human), colorectal adenocarcinoma, colon, p53 mutant: R273H/ P309S). Medium and other chemicals include McCoy's medium, DME medium, MEM, FCS, calcium phosphate transfection reagents (0.1xTE (pH8.0), 2xHBS (pH7.4), 2M CaCl<sub>2</sub>, 15% glycerol in HBS), PBS, and puromycin (Sigma).

WWP-Luc plasmid was restricted by EcoRI and SalI. The 2366bp fragment was gel-purified (1% agarose in 1x TBE) by freeze-thaw method (Wolff & Hull, 1996). Vector pUC18 was restricted by EcoRI and SalI, dephosphorylized by SAP, and purified by phenol-chloroform. Then the p21 promoter fragment was subcloned into pUC18 vector by T4 DNA ligase, generating a plasmid named as p21uc (Fig. 1a). All drug resistant markers in the constructed

plasimds, are listed in Table 1. Genes involved in the resistance are also listed.

Table 1. Drug resistant markers in constructed plasmids and their genes.

Drug	Resistant gene
Puromycin	Puromycin-N-acetyl-transferase ( <i>pac</i> )
Neomycin	Neomycin phosphotransferase gene
Hygromycin B	Hygromycin B phosphotransferase
Histidinol	Histidinol dehydrogenase ( <i>hisD</i> )

P21 promoter was cut out from p21uc first by EcoRI, blunted with Klenow and dNTP, and then by SphI. The 2450bp-p21-promoter fragment was gel-purified using the same method as above. Vector pPUR was restricted fully by PvuII and SphI, dephosphorylized by SAP, and gel-purified by the same method, which removed SV40 early promoter upstream of puromycin resistant gene. Finally the p21 promoter was cloned into pPUR vector upstream of puromycin resistant gene by T4 DNA ligase, generating the p53 reporter named as p21ur (Fig. 1b).

Verification of the construction was carried out by restriction of the generated plasmid with HindIII. The original vector pPUR has only one HindIII site thus gives one band of 4257bp after gel electrophoresis. This HindIII site was removed while constructing the reporter p21ur. Two new HindIII sites were introduced into p21ur with the p21 promoter thus can give two bands, one at 2.4kb and the other at 3.9kb (Fig. 1).

Validation of the responsiveness of p21ur to p53 was carried out by transfection of p21ur to osteosarcoma cell line U-2 OS that expresses wild type p53 protein, Saos-2 that is p53 null, and colon cell line SW480.7 that expresses R273H and P309S double mutated p53

protein. One day before transfection,  $3 \times 10^5$  U-2 OS,  $1.5 \times 10^5$  Saos-2, and  $3 \times 10^5$  SW480.7 cells were seeded to each well of a 6-well plate. Transfection was carried out by the calcium phosphate method (Parker & Stark, 1979),  
5 with  $25 \mu\text{g}$  of p21ur for each well. A 2-minute glycerol-shock was applied 3 hours after DNA was added to the cells and the transfected cells were incubated in MEM/10%FCS at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  overnight. The transfected  
10 cells were transferred to new 6-well plates at the concentration of  $5 \times 10^4$  cells per well on the next day, and incubated in growth medium (McCoy's medium/10%FCS for U-2 OS and DME medium/10% FCS for Saos-2 and SW480.7) with puromycin. The same puromycin treatment  
15 was also given to the non-transfected cells as a control. Refreshed the medium with puromycin every three-day and observed growth status of the cells.

The cloned vector was restricted by HindIII, which proved that there is the p21 promoter, a  $\sim 2.4\text{kb}$  fragment, inserted upstream of the puromycin resistant  
20 gene. DNA electrophoresis showed that the restriction gave two bands with correct theoretical sizes at 2.4 and 3.9 Kb.

Transfection of the reporter p21ur into U-2 OS cell line provided puromycin resistance to the transfected  
25 cells (Table 2). Cells transfected with p21ur kept alive after 5 days of puromycin treatment at  $0.5 \mu\text{g}/\text{ml}$ , while the untransfected cells died out. The transfected cells could not survive from higher concentration of puromycin because of the low expression level of p53 protein.  
30 Neither transfected Saos-2 cells (null p53) or SW480.7 cells (mutant p53) survived after 5 days of puromycin treatment at the concentration of  $0.5 \mu\text{g}/\text{ml}$  or higher, which proved that the resistance of p21ur-transfected U-2 OS cell to puromycin requires the existence of wild  
35 type p53 that transactivates p21 promoter. Thus it validated the proper responsiveness of the reporter p21ur to p53 in human cells.

Table 2. Validation of the reporter p21ur - its response to wild type p53 protein.

5

Puromycin ( $\mu$ g/ml)	Number of cells attached to the bottom of the well after 5 days of puromycin treatment	
	Untransfected U-2 OS	Transfected U-2 OS
0	Growing well	12600
0.5	0	11500
1	0	<5000
2	0	<5000
3	0	<5000

10

15

Example 2. Construction of peptide library expression in  
mammalian cells

20

We have constructed a microgene library to provide a series of molecular shapes for testing. This 'microgenex' approach enabled us to screen among the expressed peptide repertoire and identify some that could correct mutated p53 and could restore all of its downstream activity.

25

30

Selection of the functional peptides can be carried out either *in vivo* or *in vitro* according to the peptide library. The advantage of *in vivo* selection over *in vitro* selection is that it allows the peptides to carry out their function in an environment similar or even exactly the same as where they will later be applied in therapy. This makes the peptides more applicable and simplifies the modification step for the selected peptides. Besides, it can often detect weak and transient interactions.

35

Expression of the peptide library allows *in vivo*

screening for possible peptide ligands that can adjust  
mutant p53 protein back to its wild type function. Once  
the peptides were constructed into an expression vector,  
they were easily internalized by cells. With the help of  
5 positive-selection reporters, the possible ligands were  
also readily identified by the recovery of ligand-  
encoding plasmids from surviving cells.. Moreover,  
according to this technique, there is no need to either  
synthesize peptides or purify peptides, which can be  
10 expensive and labour intensive. An un-integrated,  
autonomously replicated mammalian expression vector was  
chosen to prevent the dilution of transfected ligand-  
encoding plasmids during cell division, or the  
integration of the plasmids into the host chromosomes,  
15 which causes difficulties in rescuing ligand from  
surviving cells.

#### Materials and Methods

pCEP4 (Invitrogen):

20 TTGACGCAAA TGGGCGGTAG GCGTGACGG TGGGAGGTCT ATATAAGCAG AGCTCGTTTA  
CAAT TATA pCEP Forward  
GTGAACCGTC AGATCTCTAG AAGCTGGGTA CCAGCTGCTA GCAAGCTTGC TAGCGGCCGC  
primer KpnI HindIII  
TCGAGGCCGG CAAGGCCGGA TCCAGACATG ATAAGATACA TTGATGAGTT TGGACAAACC  
25 EBV Reverse primer  
ACAACTAGAA

Oligo REGP-10:

30 5'-AAGAGCTCGG TACCAAGAAG GAGTTTACAT ATG GGA NNK NNK NNK TGA TAA  
KpnI M G X X X stop stop  
GGATCCAAG CTTGAATTCA G-3'  
HindIII

Oligo REGP-11:

35 5'-AAGAGCTCGGTACCAAGAAGGAG-3'  
KpnI

Oligo REGP-12:

5'-CTGAATTCAAAGCTTGGATCCTTATC-3'

HindIII

pCEP Forward primer:

5' -AGAGCTCGTTT TAGTGAACCG-3'

5

EBV Reverse primer:

5' -GTGGTTTGTCCAAACTCATC-3'

---

10    ~~KpnI, HindIII, T4 DNA ligase, 10xT4 DNA ligase buffer~~

---

15    Oligo REGP-10 was used as a template, while oligos REGP-11 and REGP-12 were used as forward and reverse primers, to generate double-stranded DNA fragments encoding a degenerated peptide library by PCR. The first ATG in this fragment encodes Met as the translation initiation site of the peptides. Gly, the second amino acid encoding by the DNA fragment, assures the peptides expressed are stable inside cells according to the N-end rule (Varshavsky, 1997). The following three repeats of NNK (N for A, C, G, and T in equal molar ratio, K for G and T in equal molar ratio) code for all possible amino acids and thus form the peptide library. Two stop codons, TGA and TAA, are added right after the last NNK to stop peptide translation. The 5'-end KpnI site and 3'-end HindIII allows the fragments to be cloned into pCEP4 vector in the correct direction under CMV promoter.

30    After ligation of KpnI/HindIII restricted PCR product and KpnI/HindIII restricted pCEP4, the DNA molecules were electroporated into XL1-blue cells and plated on LB plates with ampicilin. All colonies from the plates were poured together, delivered into small portions and stored at - 70°C. Each portion of the transformed cells can be inoculated and start a 1-liter-culture in order to prepared the plasmids by Qiagen Maxi-Prep kit. Insertions were verified by PCR of the generated

plasmids with oligos REGPD-11 and REGPD-12. Same PCR reactions were also applied to plasmids prepared from 6 randomly picked colonies so as to estimate the cloning efficiency and the ratio of peptide-encoding plasmids to empty vectors in the population. Note that all generated peptides will start with M and G amino acids followed by three random amino acids.

### Results

There were ~25000 colonies on the plates. They were collected into 12 portions. Plasmid prepared from one of such portions was examined by PCR.

Gel electrophoresis showed that there were insertions with correct size. PCR results from the 6 randomly picked colonies from the original library plates showed that 4 of the colonies had the right construction (showed correct size of insertion) while two did not. The structure of the generated peptide library plasmid is shown in Figure 2.

### Example 3. Screening for ligands that can correct p53 mutants in SW480.7 cells

SW480.7 is a human colon cancer cell line that carries R273H and P309S double mutations in its p53 gene. R273H is one of the most frequently occurred p53 mutations that have been observed in various cancer tissues (Sigal & Rotter, 2000). It has been reported that the p53 mutant in SW480.7 loses its function in transactivating its downstream genes such as p21 (El-Deiry et al., 1993), Bax (Yu et al., 1999), 14-3-3 $\sigma$  (Hermeking et al., 1997), and CD95 (Fukazawa et al., 1999). Our purpose of screening the peptide library constructed above is to find out one or more peptide ligands that can restore wild-type p53 function without alteration of the mutant

p53 gene in the cell and disturbing cells with wild type p53. The choice of a human cancer cell line provides the real human environment in which the selected peptides can be modified, degraded, and transported to  
5 subcellular organelles, and thus assures the reliability of the selected peptides to function in human body.

### Materials and Methods

---

~~Human colon cancer cell line SW480.7 with p53 mutations~~

10 R273H and P309S (ATCC Number: CCL-228, Homo sapiens (human), adenocarcinoma, colorectal; colon), DMEM medium, FCS, PBS, trypsin, puromycin (sigma), hygromycin (sigma), Peptide library (constructed by our laboratory described as above), pCEP4 vector (Invitrogen), calcium  
15 phosphate transfection reagents (0.1xTE (pH8.0), 2xHBS (pH7.4), 2M CaCl<sub>2</sub>, 15% glycerol in HBS).

DNA extraction reagents (TBS buffer (0.8% NaCl, 0.02%KCl, 0.3%Tris, pH7.4), TE buffer (pH8.0), DNA  
20 extraction buffer (10mM TrisCl, 100mM EDTA, 20µg/ml RNase, 0.5% SDS, pH8.0), Proteinase K (18mg/ml), chloroform, phenol (Tris saturated), ethanol, 7.5M ammonium acetate (pH 7.4)), XL1-blue electroporation competent cell, pCEP Forward primer, EBV Reverse primer,  
25 dye-terminator thermocyclic DNA sequencing kit, ALF express DNA automatic sequencing system.

The peptide library was transfected into SW480.7 using the calcium phosphate method described in the reporter  
30 constructing part. Meanwhile pCEP4 was also transfected into the SW480.7 cells as a negative control. On the second day after transfection, puromycin was added into the medium at the concentration of 0.5µg/ml. Then the transfected cells were washed with PBS and changed with  
35 fresh medium with 0.5µg/ml puromycin every three days. After all the negative control cells died, all the library-transfected cells that still attached to the

wells were collected by scraping into ice-cold TBS buffer.

5 Rescue of library plasmids was carried out by the  
extraction of total DNA from the collected puromycin  
resistant cells according to Blin and Stafford's method  
(Blin & Stafford, 1976). The extracted DNA was then  
directly electroporated into the competent cell XL1-blue  
and plated on LB plates with ampicillin. All the  
10 colonies were picked up for plasmid preparation. The  
plasmids were first screened by size using agarose gel  
electrophoresis to get rid of the reporters. The rest of  
the plasmids were sequenced by ALF express DNA automatic  
sequencing system (Pharmacia) using EBV Reverse  
15 sequencing primer (Invitrogen) to reveal the DNA  
sequence, hence amino acid sequence of the selected  
peptide ligands.

### Results

20 No pCEP4-transfected cells survived in the well after 7  
days of selection with puromycin, while there were still  
some living library-transfected cells sticking to the  
bottom of the well.

25 Electroporation of the DNA extracted from the library-  
transfected cells after 7 days of selection gave ~150  
colonies. Among them, 50 of the colonies have the right  
size of library plasmid and were sequenced. One ligand  
peptide sequence was found. The DNA and amino acid  
30 sequences are:

DNA: 5'-GGTACCAAGAAGGAGTTTACATATG GGA TGG TGT ACT TGA TAA

KpnI

Amino acid: M G W C T stop stop

5 DNA: GGATCCAAGCTT-3'  
HindIII

10 Example 4. Validation of the selected peptide ligand

The purpose of this Example is to confirm that the selected peptide ligand indeed restores the mutated p53 protein to the wild-type p53 function in SW480.7 cells.

15 The selection of peptide ligands was based on puromycin resistance. There might be a chance that the cells got the resistance with, or even without the help of the selected ligand via mechanisms other than p53-transactivated p21 promoter. Thus we should make sure  
20 that the mutant p53 in SW480.7 cells functions as wild-type p53 only if the selected ligand is added. Confirmation/validation was achieved in two different ways:

25 1- We observed the function of the peptide ligand on cell cycle control of SW480.7 cells, as well as that of U-2 OS (WTp53) and Saos2 cells (no p53 copy). The selected peptide ligand can be further verified only if it can induce cell  
30 cycle arrest or apoptosis in SW480.7 cells but neither in U-2 OS nor in Saos2 cells, which means the specificity of the selected ligand to p53 mutation in SW480.7.

35 2- We also tested the transcription levels of genes that are only transactivated by wild-type p53. If the transcription levels of these genes are highly induced in ligand treated cells compared with the negative control (cells transfected  
40 only with the empty vector (pCEP4). The selected

ligand/ peptide was proved to be capable of altering the mutant p53 function in SW480.7 into wild-type function.

5 P53 (wild-type) regulates different cell responses via transactivating different downstream genes. For example p53 causes cell cycle arrest at G1 phase by transactivation of p21 gene. While p53 causes cell arrest at G2 phase by transactivation of CD95 gene.  
10 Apoptosis is due to p53 transactivation of 14-3-3 $\sigma$  and/or Bax gene (Vogelstein et al., 2000; Xu & El-Gewely, 2001). Thus we measured p21, CD95, and 14-3-3 $\sigma$  gene transcription levels by RT PCR, to reveal the capability of the selected peptide ligand in restoring  
15 the wild-type p53 transactivation of the downstream genes. The expression level of  $\beta$ -actin was measured as a control.  $\beta$ -Actin transcription level was used to normalize total mRNA amount between samples for comparison owing to its constitutive expression in all  
20 cells.

#### Materials and Methods

SW480.7, U-2 OS, Saos 2, DMEM, McCoy's medium, FCS, PBS, trypsin, trypan blue (Sigma), calcium phosphate  
25 transfection reagents, peptide ligand (selected above), pCEP4 (Invitrogen), TRIZOL<sup>®</sup> Reagent, chloroform, isopropyl alcohol, 75% ethanol (in DEPC-treated water), 0.01%(v/v) DEPC water, SuperScriptII reverse transcriptase (Life Technology), TE buffer (10 mM Tris  
30 (pH7.6), 1 mM EDTA), ethanol, 4M ammonium acetate (pH7.0), 1-kb DNA ladder, 10 x TBE electrophoresis buffer, 1% agarose in 1xTBE buffer with ethidium bromide.

35 Primers for RT-PCR are listed in Table 3.

Table 3. RT-PCR primer sequences.

	Name	Sequence
	RT-WAF1-FOR	5'-CTACCTCAGGCAGCTCAAGC-3'
	RT-HME1-FOR	5'-AGACAGCACCCCTCATCATGC-3'
5	RT-CD95-FOR	5'-TGGTGCTCATCTTAATGGCC-3'
	RT-ACTIN-FOR	5'-TGACAAAACCTAACTTGCGC-3'
	CDS Primer	5'-AAGCAGTGGTAACAACGCAGAGTACT <sub>(30)</sub> N <sub>-1</sub> N-3'
10	PCR Primer	5'-AAGCAGTGGTAACAACGCAGAGT-3'

Target genes and their corresponding RT-PCR product using the above primers are listed in Table 4.

Table 4. RT-PCR primer target genes and their corresponding product.

Primer name	Target gene		Amplify region in target mRNA	Size of RT-PCR product
	Name	Function		
20 RTWAF1FOR	p21	G1 arrest	1514-2121	607 bp
RTHME1FOR	14-3-3 $\sigma$	G2 arrest	652-1245	593 bp
25 RTCD95FOR	CD95	Apoptosis	1968-2534	566 bp
RTACTINFOR	$\beta$ -actin	cytoskeleton	1236-1793	557 bp

Cells SW480.7, U-2OS, and Sa-os2 were transfected with peptide ligand and pCEP4 parallelly using the calcium phosphate method. The transfection plan was shown in Table 5:

Table 5. Transfection plan for the verification of peptide ligand function.

Cell type	SW480.7		U-2OS		Sa-os2	
DNA	Ligand	pCEP4	Ligand	pCEP4	Ligand	pCEP4
Number of 6-well plates	3	3	1	1	1	1

After transfection, cells were cultured without drug selection and diluted at the ratio of 1:100 every five days. Cell proliferation status was closely observed until ligand transfected SW480.7 cells died out. Life staining with 0.4% trypan blue was applied to determine the death of the cells. Dead cells can not exclude the dye thus were stained blue. Living cells can pump the dye outside thus were not stained.

Total RNA was collected from two wells of one ligand transfected SW480.7 plate and one pCEP4 transfected SW480.7 plate each day on day 0, day 1, and day 2 after transfection. Thus RNA samples were harvested as SW480.7/Ligand-0day, SW480.7/Ligand-1day, SW480.7/Ligand-2day, SW480.7/pCEP4-0day, SW480.7/pCEP4-1day, SW480.7/pCEP4-2day. Protocol for total RNA extraction by TRIZOL® Reagent was according to that given by Life Technology:

#### 1. Homogenization

Wash the cell with DEPC water once, then add 1ml of TRIZOL Reagent per well. Pass the cell lysate several times through a pipette.

#### 2. Phase separation

Incubate the homogenized samples for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Add 200 µl of chloroform per 1 ml of TRIZOL Reagent. Cap the sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at room temperature for 2 to 3 minutes.

Centrifuge the samples at no more than 12,000 x g for 15 minutes at 2 to 8°C.

### 3. RNA precipitation

5      Transfer the aqueous phase to a fresh tube. Precipitate the RNA by adding 500 µl of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at room temperature for 10 minutes and centrifuge at no more than 12,000 x g for 10 minutes at 2 to 8°C.

---

### 10      4. RNA wash

Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the sample by vortexing and  
15      centrifuge at no more than 7,500 x g for 5 minutes at 2 to 8°C.

### 5. Re-dissolving the RNA

Briefly dry the RNA pellet. Dissolve RNA in DEPC water by passing the solution several times through a pipette  
20      tip, and incubation for 10 minutes at 55 to 60°C. Determine the concentration by measure OD<sub>260</sub>. Store at -70°C.

### RT-PCR:

#### 25      First-Strand cDNA Synthesis

1.      For each RNA sample, combine the following reagents in a sterile 0.2-ml reaction tube:

	1-3 µl RNA sample
	(using same amount of RNA among
	samples)
30	1 µl CDS primer (10 µM)
	1 µl RT forward primer (10 µM)
	x µl Deionized H <sub>2</sub> O
	<hr/> 5 µl Total volume

35      2. Mix and spin the tube briefly.

3. Incubate the tube at 70°C in a thermal cycler for 2 min.

4. Spin the tube briefly to collect contents at the

bottom. Keep tube at room temperature.

5. Add the following to each reaction tube:

- 5      2     $\mu$ l   5 x First-Strand Buffer  
        1     $\mu$ l   DTT (20 mM)  
        1     $\mu$ l   50 x dNTP (10 mM)  
        1     $\mu$ l   SuperScripII reverse  
                  transcriptase (200 units/ $\mu$ l)

6. Gently vortex and spin the tubes briefly.

10      7. Incubate the tubes at 42°C for 1 hr in an air  
          incubator or cycler.

8. Add 40  $\mu$ l TE buffer (10 mM Tris (pH7.6), 1 mM  
    EDTA) to dilute the first-strand reaction product.

9. Heat tubes at 72°C for 7 min to inactivate reverse  
    transcriptase.

15      10. Samples can be stored at -20°C for up to three  
          months.

#### cdNA Amplification by PCR

1. Preheat the PCR thermal cycler to 95°C.

20      2. For each reaction, 5  $\mu$ l of diluted first-strand  
          cdNA and 5  $\mu$ l of deionized water are added to a  
          labeled 0.2-ml reaction tube.

3. Prepare a master mix for all reaction tubes,  
    plus one additional tube. For each reaction:

25	20.5	$\mu$ l	Deionized water
	5	$\mu$ l	10 x PCR buffer
	1	$\mu$ l	50 x dNTP (10 mM)
	1.5	$\mu$ l	PCR primer (10 $\mu$ M)
	1.5	$\mu$ l	RT forward primer (10 $\mu$ M)
30	10	$\mu$ l	5x Q solution
	0.5	$\mu$ l	HotStart Taq DNA polymerase
<hr/>			
	40	$\mu$ l	Total volume

4. Mix by vortexing and spin the tube briefly.

35      5. Aliquot 40  $\mu$ l of the PCR Master Mix into each tube  
          from Step 2.

6. Cap the tube, and place it in the preheated thermal cycler.

7. PCR was carried out using the following cycling parameters:

5	95 °C	15 min
	2 cycles	
	94 °C	30 sec
	70 °C	30 sec
	72 °C	1 min
10	2 cycles	
	94 °C	30 sec
	69 °C	30 sec
	72 °C	1 min
	2 cycles	
15	94 °C	30 sec
	67 °C	30 sec
	72 °C	1 min
	2 cycles	
20	94 °C	30 sec
	65 °C	30 sec
	72 °C	1 min
	2 cycles	
	94 °C	30 sec
	63 °C	30 sec
25	72 °C	1 min
	2 cycles	
	94 °C	30 sec
	61 °C	30 sec
	72 °C	1 min
30	2 cycles	
	94 °C	30 sec
	59 °C	30 sec
	72 °C	1 min
	2 cycles	
35	94 °C	30 sec
	57 °C	30 sec
	72 °C	1 min
	40 cycles	
40	94 °C	30 sec
	55 °C	30 sec
	72 °C	1 min
	1 cycle	
	72 °C	7 min
45	4 °C	store

8. Electrophoreses 5 µl of each PCR reaction alongside 0.1 µg of 1-kb DNA ladder on a 1.2% agarose/EtBr gel in 1 x TBE buffer.

## Results

After transfecting the ligand into SW480.7 cells for 15 days without puromycin selection, the cells could no longer attach to the wall and died. On the contrary, the  
5 ligand-transfected U2-OS and Saos-2 cells were growing well.

RT-PCR analysis of the mRNA from ligand transfected SW480.7 cells showed that this ligand can reestablish  
10 wild type p53 function. Higher mRNA levels of p21 (WAF1/Cip1), 14-3-3 $\sigma$  and CD95 genes were found compared with that in the empty pCEP4 transfected SW480.7 cells (Figure 4). This proved that the apoptosis and cell  
15 growth arrest of ligand transfected cells were owing to the ligand that corrected R273H/P309S p53 mutant to wild type function.

## Example 5. New reporter system

20 The previous p53 reporter p21<sub>lur</sub> is based on the transactivation activity of wild type p53 to p21 promoter. It has been proved that up-regulation of p21 protein expression will result in cell growth arrest, but  
25 not apoptosis. Another p53 target, Bax, can lead to apoptosis once its transcription and expression is upregulated. Bax is one of the key mediators for p53 to induce cell apoptosis - to kill the cells that have failed to repair their damaged genomes (Vogelstein et  
30 al., 2000; Xu & El-Gewely, 2001).

Bax promoter is different from p21 promoter, though both have p53 consensus binding sequence. It has been reported that some p53 mutant can transactivate p21 promoter, but  
35 not Bax. This indicates a more stringent requirement of p53 conformation for the transactivation of Bax promoter (Thornborrow & Manfredi, 1999).

Green fluorescence protein (GFP) has also been used in the new reporter system. Bax promoter drives a long transcript of both puromycin resistant gene and GFP gene. By inserting an IRES fragment between the two genes that  
5 introduces a second ribosome-binding site, they can be translated into protein simultaneously from one transcript (Clontech pIRESneo manual). Thus the green color from GFP will visualize the Bax promoter activity, hence the ligand's activity to correct p53 mutants, in

10 addition to the positive selection of puromycin resistance. The green fluorescence will eliminate the background caused by mechanisms which have cells developed to survive from puromycin selection other than transactivation of Bax promoter.

15 Selection marker is important for integration of the plasmid into genome. A stable p53-null cell line with integrated reporter system will be convenient for ligand screening, especially for non-peptide ligands.

#### 20 **Materials and Methods**

Plasmids used in this Example are: p21ur (above), pIRESneo (Clontech), pEGFP-C1 (Clontech), and pREP8 (Invitrogen).

25 Genomic DNA (human, male, normal)

Restriction endonucleases (Promega): AgeI, EcoRV, HindIII, NruI, PvuI, SacI, SalI, SpeI, XbaI, XhoI, XmaI.

30 Modification enzymes: Vent DNA polymerase (NEB), T4 DNA ligase (Promega), and Shrimp alkaline phosphatase (SAP).

Bax promoter primer:

Bax promoter Forward:

35 5'-atctaaagcttgaggcttcagcccggaattccag-3'  
HindIII

Bax promoter Reverse:

5'-atctaccggtgccagcagtggcgccgtccaacag-3'  
AgeI

EGFP primer:

5 EGFP Forward: 5'-aataa cccgGGTCGCCACCATGGTGAGCAAG-3'  
XmaI

EGFP Reverse: 5'-aataa tctagaACTTGTACAGCTCGTCCATGCCG-3'  
XbaI  
stop

10

Construction of pBaxur - Bax promoter controlled puromycin resistant gene (Figure 5.):

- 15 • Making PCR from normal human genomic DNA using Bax promoter primers. The PCR product is 500bp. It has a SmaI site near its 5'-end and a SacI site near its 3'-end.
- Cut PCR product Bax promoter with HindIII and AgeI.
- Cut p21ur with HindIII and AgeI, and dephosphorylate afterwards. Purify the 3,9kb fragment from gel.
- 20 • Ligate Bax promoter into the 3,9kb fragment so that Bax promoter can control the puromycin resistant gene expression in mammalian cells. The final product is denoted as pBaxur.

25

Construction of pBaxurEGFP and p21urEGFP - Green fluorescent protein downstream of puromycin resistant gene that visualizes the transcription level of Bax or p21 promoter (Figures 6, 7):

- 30 • Amplify EGFP gene fragment from pEGFP-C1 vector by PCR with the EGFP primers.
- The PCR product is around 750bp. Cut the product with XmaI and XbaI, then purify it from gel.
- Cut pIRESneo with XmaI and XbaI, then purify the 4,5kb fragment from gel.
- 35 • Ligate PCR product with pIRESneo 4,5kb fragment to generate vector pIRES-EGFP (5,2kb).
- Cut p21ur or pBaxur with EcoRV and XbaI, and purify the 3.2kb or 1.3kb fragment from gel.
- 40 • Cut pIRES-EGFP with NruI and SpeI, dephosphorylate, and purify the 4.4kb fragment from gel.

- Ligate the two fragments to generate transient p53-double-reporter p21urEGFP (7.6kb) or pBaxurEGFP (5.7kb).

Construction of pBaxurEGFP-hisD - Integrative plasmid with selection mark as histidinol D resistant (Figure 8):

- Cut pREP8 with PvuI, SalI, and SacI, purify the 5194bp fragment from gel.
- Cut pBaxEGFP with PvuI and XhoI, dephosphorylated, and purify the 3972bp fragment from gel.
- Ligate the hisD fragment into pBaxurEGFP fragment to generate integrative p53-double reporter pBaxurEGFP\_hisD.

The structure of p21urEGFP was verified by restriction of HindIII, which should give five bands of 3489bp, 2339bp, 1041bp, 360bp, and 348bp. Agarose gel electrophoresis of the restricted plasmid showed the expected bands.

The structure of pBaxur was verified by restriction of either HindIII together with SacI, which should give two bands of 3996bp, and 388bp, or SmaI alone, which should give three bands of 3184bp, 620bp, and 580bp. Agarose gel electrophoresis of the restricted plasmid showed the expected bands.

The structure of pBaxurEGFP was verified by restriction of either EcoRI together with XbaI, which should give two bands of 2907bp, and 2766bp, or SacI alone, which should give two bands of 3125bp, and 2548bp. Agarose gel electrophoresis of the restricted plasmid showed the expected bands.

The structure of pBaxurEGFP-hisD was verified by restriction of either EcoRI, which should give two bands of 5955bp, and 3211bp, or SacI, which should give two bands of 6618bp, and 2548bp. Agarose gel electrophoresis of the restricted plasmid showed the expected bands.

Screening for new peptide ligands is done by first transfecting the outlined new reporter p21urEGFP-hisD along with the cotransfection of the peptide library constructed above into cancer cell lines such as SW480.7. Caspase inhibitor VAD-fmk (ApoAlert®, Clontech) is added to the cell culture at the final concentration of 40mM in order to prevent apoptosis in the case of the overexpression of BAX gene. Also 0.5µg/ml puromycin is added in the media one-day after transfection. After several days, surviving green cells (excited at 488nm wavelength) will be the cells containing the candidate ligands. Rescued plasmids from these cells are subjected to DNA sequencing to deduce the amino acid sequences of the encoded peptide ligands.

When screening non-peptide ligands, a stable cell line of the reporter p21urEGFP-hisD is constructed. Members of the chemical compound library are added to the cell culture. The cells should also be cultured in the medium containing 40mM VAD-fmk and 0.5µg/ml puromycin. Surviving green cells after excitation at 488nm is the indicator for successful candidate ligands.

Example 6 : Modulation of the *E. coli* tryptophan repressor by expressed peptides in vivo

The *E. coli* Tryptophan repressor protein is 107 amino acid, dimeric helix-turn-helix DNA-binding protein. TrpR is a global regulator involved in the transcriptional regulation of 5 unlinked operator regions: *trp* EDCBA, *aroH*, *trpR*, *aroL* and *mtr*. TrpR regulates both the import of tryptophan (*mtr*), and the first step in the tryptophan biosynthesis (*trpE*). Moreover, TrpR regulates two genes within the common aromatic biosynthetic pathway (*aroH* and *aroL*), in addition to itself.

In this example we investigate whether a strong

negatively complementing TrpR mutant such as T44M could be modulated *in vivo* by expressed ligands, selected from a pool of expressed ligands. Thereby, restoring the DNA binding function of a non-operative *E. coli* TrpR(T44M) mutant protein without genetically modifying either the protein, or its cognate DNA target.

In order to select and verify our selected peptide-ligands, a PheS survival reporter system and confirmatory b-galactosidase reporter was used. Several additional experiments were conducted to verify modulation.

#### Materials and Methods:

##### Table 1 Primers used in Experiment

15	Trp168 Forward:	TCGTAAATCACTGCATAATTCG
	Trp168 Reverse:	GTCCATACCCTTTTACGTGAA
	PBad Forward:	GATTAGCGGATCCTACCTGACG
	pBad Reverse:	GCCAGGCAAATTCTGTTTTATC
	T44M forward:	TCAGGTCGGAATTATCGCATTAT
20	T44M Reverse:	TCGCCGTAATGGCTAGTCACATCC
	T7 Forward :	TAATACGACTCACTATAGGG
	T3 Forward :	ATTAACCCTCACTAAAG
	TrpG85E forward:	CGATTACGCGTGAATCTAACAGCC
	TrpG85E reverse:	GGCTGTTAGATTACGCGTAATCG
25	TrpT44M forward:	CCTGATGCTGATGCCAGATGAGCGC
	TrpT44M reverse:	GCGCTCATCTGGCATCAGCATCAGG
	PheS forward:	GATAATGTGCGCGTCGAATA
	PheS reverse:	TTTGCGGAAACGCAGATCGT
	Amp forward:	GTGTCGCCCTTATTCCCTTT
30	Amp reverse:	GGCACCTCTCTCAGCGATCT
	LacZ forward:	AGCGAATACGTCTTCCGTCA
	LacZ reverse:	GATGGCTGGTTTCCATCAGT

##### Table 2 Media used in Experiment:

YE: (5g yeast extract, 10g NaCl, 1mM tryptophan, Ampicillin 200 ug/ml, add H<sub>2</sub>O to 1 litre)  
YEA: (YE with 0.2% Arabinose, 0.2% CAA)

YEG: (YE with 0.2% Glucose)

YEPG: (YEG with 2g DL-p-Cl-phenylalanine [®Sigma, St. Louis, Mo. USA])

YEPA: (YEA with 2g DL-p-Cl-phenylalanine)

5 LB: (10g Bacto Tryptone, 5g Bacto Yeast Extract, 10g NaCl)

BTTR: (10g bacto-tryptone, 5g NaCl, X-gal 40µg/ml, 1mM tryptophan, phenylethyl-βD-galactopyranoside [®Sigma, St. Louis, Mo. USA], dH<sub>2</sub>O to 1L)

10 BTTRA: (BTTR with 200µg/ml Ampicillin, 2g/L DL-p-Cl-phenylalanine, 2% Arabinose)

BTTRG: (BTTR with 200µg/ml Ampicillin, 2g/L DL-p-Cl-phenylalanine, 2% Glucose)

Bactotryptone plates: (BTTRA of BTTRG with 15g agar/L)

15

Table 3 Solutions used in Experiment:

Sample Buffer I: (0.3% SDS, 200 mM DTT, 50 mM Tris 8.0, protein inhibitor cocktail of choice)

20 Sample Buffer II: (500 mM Tris pH 8.0, 50 mM MgCl<sub>2</sub>, 1mg/ml DNaseI, 0.25 mg/ml RNase A, or 0.1 mg/ml Benzonaze instead of (DNA/RNase)).

25 Rehydration: (8mol /L Urea 24g, 0.5% CHAPS 250mg, 0.25% Pharmalytes 3-10 310ul, 0.2%DTT 100mg, 0.01% Bromophenol blue 1 mg, add water to 50 ml)

ESS: (2% SDS 2.0g, 6mol/L Urea 36g, 0.1mmol/L EDTA, 3mg, 0,01% Bromophenol Blue 10mg, 50 mmol/L trisHCl pH 6.8 10ml, 30% Glycerol (v/v) 35ml of 87% solution, add 100 ml dH<sub>2</sub>O)

30 Sample Application Buffer: (Urea 8M, CHAPS 4% w/v, Tris 40mm pH 8.0, DTT 65mm)

Cathode buffer 10x: (Tris 30.4g, Glycine 144g, SDS 10g, in 1L dH<sub>2</sub>O)

35 Anode buffer 10x: (Tris 30.4g, fill with dH<sub>2</sub>O to 800ml, titrate with 4M HCl to pH 8.4, fill to 1L)

Light 8% solution: (50ml acrylamide 40%(37.5:1), 145ml dH<sub>2</sub>O, 75ml 4 x slab buffer, 30ml glycerol, 300 µl TEMED)

Heavy 16% solution: (115ml acrylamide 40%(37.5:1), 35ml dH<sub>2</sub>O, 75ml 4 x slab buffer, 75ml glycerol, 300 ul TEMED)

Slab buffer 4x: (Tris 181.8g, fill with dH<sub>2</sub>O to 800ml, titrate with 4M HCl to pH 8.8, fill to 1L)

- 5 SDS lysis solution: 2% SDS, 16mM EDTA (add 200mM NaCl if cells are grown in low salt media like LB).

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Table 4 Cell Lines and Plasmids used in Experiment

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- 10  $\lambda$ RTL<sup>F</sup> - W3110, tnaA2, trpR2,  $\Delta$ lac169, [ $\lambda$ , trpOP, trpL'-trpE'-lacZ', lacY,  $\lambda$ ] (Paluh, J.L. et al. Nucleic Acids Res. (1986) 14: 7851-60)
- XL1-Blue\*- recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F' proAB lacI<sup>q</sup>Z $\Delta$  M15 Tn10 (Tet<sup>r</sup>)]<sup>c</sup>
- 15 pBAD33- cam<sup>r</sup>, p15a-origin, AraC, pBAD (Guzman, L.M. et al. J. Bacteriol (1995) 177: 4121-30)
- pTrpRep- amp<sup>r</sup>, pMB1-origin, TrpEDCBA•OP'-pheS, tet•OP'-trpR (current study)
- pKSS- amp<sup>r</sup>, pUC-origin, lacI, lacZop, fl, pheS\*(Ala-Gly294) (Kast, P. Gene (1994) 138: 109-114)
- 20 \*Stratagene, 11011 North Torrey Pines Road, La Jolla, CA 92037

Construction of the pTrpRep(None) Reporter:

- 25 *E. coli* genomic DNA was extracted using standard procedures and used as a template along with the Trp168 primers (Table 1) to initiate the following PCR reaction resulting in a 168bp fragment. PCR reaction : Trp168 forward and reverse primers 20 mm, template 500 ng, MgCl<sub>2</sub> 3mM, dNTP 0.2 mM, 1 unit Pfu turbo<sup>TM</sup> (Stratagene), 5  $\mu$ l
- 30 10 x reaction buffer, dH<sub>2</sub>O to total 50  $\mu$ l. The 168bp PCR fragment containing the TrpEDCBA promoter operator was cleaned using the Qia quick PCR cleanup kit. Two microliters of pKSS vector was cut with ClaI restriction enzyme for 90 minutes at 37°C according to the
- 35 manufactures instruction. ClaI restriction was in Promega buffer C. Following ClaI restriction, 1 unit of T4 DNA polymerase and 0.2 mM dNTP were added directly to the reaction and the sample and incubated at 37°C for an additional 10 minutes. The entire 168bp fragment was then

purified from an agarose gel using the Qia quick clean kit. Two hundred nanograms of purified *Cla*I cut, T4 flush ended, pKSS vector was added to 200 nanogram of the 168bp *TrpEDCBA* PCR fragment along with 40 units of T4 DNA  
5 ligase and ligase buffer in a total of 8  $\mu$ l and incubated overnight at 18°C. Two microlitres of the ligation reaction were mixed with XL1 competent cells prepared using Inoue et al 1990, Gene 96, 23-28 and plated out on LB plates with 0.2% glucose and 200 $\mu$ g/ml ampicillin.  
10 Verification of 168bp DNA fragment and fragment orientation was by *spe*I restriction enzyme digestion. DNA sequence verification was done using the T3 forward primer. The resulting plasmid with the PheS gene under the transcriptional control of the *TrpEDCBA* operator  
15 promoter was called pTrpRep(None)

Construction of the pTrpRep(WT) Reporter:

The constitutive tet promoter and TrpR protein-encoding fragment was cut using enzymes *Xba*I and *Sph*I from  
20 pACYC184-*trp*WT (Storbakk, N., C. et al. J. Mol. Biol. 256:889, 896). The 1141 bp fragment was blunted as above and gel-purified. Two microlitres of pKSS vector containing the *TrpEDCBA* operator fragment was digested with *Not*I and subjected to the DNA overhangs filled with  
25 T4 polymerase using standard methods. The pTrpRep(None) vector was then gel purified as above. Approximately, 200ng of the pTrpRep(None) vector was mixed with 500 ng of 1141bp tet-*TrpEDCBA* operator fragment along with 40 units of T4 DNA ligase and incubated overnight at 18°C.  
30 Competent lRTLF cells were transformed with 2 $\mu$ l of the ligation mix and plated out on LB media with 0.2% glucose and 200  $\mu$ g/ml ampicillin. Confirmation of insert was by blue/white screening on bactotryptone plates (Table 3), by restriction enzyme analysis and by sequencing using T3  
35 and T7 primers (Table 1). The verified vector construct was called pTrpRep(WT).

Construction of pTrpRep(T44M) and pTrpRep(G85E):

The wild type gene encoding for the tryptophan repressor protein was mutated by Stratagene's Quick-change™  
5 site-directed mutagenesis kit to introduce a methionine residue at the 44 threonine position and a glutamate residue at the glycine 85 position. Primers used for mutagenesis are listed in Table 1: G85E forward, G85E  
reverse, T44M forward, T44M reverse. Sequence  
10 verifications were through DNA sequencing using the T7 and T3 primers.

Construction of pPepLib:

A peptide expression library was constructed to express  
15 under the arabinose promoter of the pBAD33 plasmid. For details see Example 7 herein.

Reporter system verification:

Two 50 milliliters cultures of  $\lambda$ RTL<sup>+</sup> cells containing  
20 either pTrpRep(WT) or pTrpRep(T44M) vector plasmids were grown in LB media at 30°C with shaking to a density of 0.6 at wavelength 600nm. Cells were diluted 1 to 5000 in the YE media. A 250 $\mu$ l aliquot of the two diluted cultures were then plated onto YE plates with 0.2% glucose, with  
25 or without 1 mM tryptophan (+/- W) and with or without DL-p-Cl-phenylalanine(+/- P) at 2g per liter. An overview as well as results can be seen in Table 4.

Isolation of the active peptide ligand for pTrpRep(T44M):

30 DNA was isolated from 50ml cultures of surviving white TrpT44Mrep/pPepLib colonies on YEPA plates using the Quiagen midiprep kit™ and protocol. The DNA was then restricted for 90 minutes at 37°C with restriction enzyme speI which cuts only in the pTrpRep(T44M) vector and

hopefully not in pPepLib. Competent XL1 cells were transformed with the restricted DNA and plated out on to LB plates with 25 $\mu$ g/ml chloramphenicol. Consequent colonies were then replica-plated onto LB plates with 25 $\mu$ g/ml chloramphenicol as well as plates with 200 $\mu$ l/ml ampicillin. Colonies that grew in the presence of chloramphenicol but not ampicillin were assumed to have lost the TrpT44M vector.

10 Selection of an active microgene:

$\lambda$ RTL cells containing the pTrpRep(WT) were transformed with pPepLib and the pBAD33 control vector. Likewise,  $\lambda$ RTL cells containing the TrpRep(T44M) reporter constructs were transformed with pPepLib and the pBAD33 control vector. Cells were grown in LB media at 30°C with shaking to an OD 0.6 at wavelength 600nm. Cells were diluted 1 to 5000 in the media (YEG or YEA with 25 $\mu$ g/ml chloramphenicol) and 250 $\mu$ l plated out on plates (YEG, YEPG, YEA, YEPA, with 25 $\mu$ g/ml chloramphenicol). All surviving white colonies from the TrpT44Mrep/pPepLib transformation on YEPA plates were harvested and transferred to LB media with 25 $\mu$ g/ml chloramphenicol for amplification and DNA isolation.

25 Re-transformation of the rescued pPepLib peptide:

One microliter of the isolated pPepLib (100 ng), and 1 $\mu$ l of pBAD33 (100ng), were transformed into a fresh  $\lambda$ RTL culture containing the TrpRep(T44M) reporter vector were plated out on to YEPA plates 25 $\mu$ g/ml chloramphenicol. Plates that showed an abundance of white colonies were assumed to code for a peptide that restored TrpR DNA binding function. The pPepLib plasmid was sequenced using Amersham Alpha express automatic sequencer and Cy5 dye terminator kit. Primers used in the sequencing reaction were pBad forward and pBad reverse primers (Table 1).

β-Galactosidase:

A λRTL culture containing pTrpRep(WT), pTrpRep(T44M), pTrpRepG85E, and pTrpRep(None) were transformed with one of  
5 pPep(MGFLR) or pPep(MGFWR), and grown in both BTTRG and BTTRA media to an OD of 0.6 at wavelength 600 at 30°C with shaking. Two hundred microliters of cultures assayed for β-galactosidase levels at wavelength 405nm as described in Fenton et al(1998) BBRC 242(1):71-78.

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10 λRTL cultures containing one of pTrpRep(WT), pTrpRep(T44M), pTrpRepG85E, or pTrpRep(None) were grown in BTTRG media supplemented with one of following synthesized peptides MGFLR, MGFWR, MGRRW, FLR, FWR at a concentration of 1mM. The cultures were grown to an OD of  
15 0.6 at wavelength 600 at 30°C with shaking. Two hundred microlitres of cultures assayed for β-galactosidase levels at wavelength 405 as described in Fenton et al(1998) supra.

20 Effect of Tryptophan corepressor on β-galactosidase values with plasmids:

A λRTL culture containing pTrpRep(WT), pTrpRep(T44M), pTrpRepG85E, and pTrpRep(None) were transformed with one of  
25 pPep(MGFLR) or pPep(MGFWR), and grown in BTTRA media with and without tryptophan to an OD of 0.6 at wavelength 600 at 30°C with shaking. Two hundred microliters of cultures assayed for β-galactosidase levels at wavelength 405 as described in Fenton et al (1998) supra.

30 Effect of Tryptophan on β-galactosidase values with plasmids:

λRTL cultures containing pTrpRep(T44M) were grown in BTTRG media, with and without tryptophan, and supplemented with one of following synthesized peptides

MGFLR, MGFWR, MGRRW, FLR, FWR at a concentration of 1mM. The cultures were grown to an OD of 0.6 at wavelength 600 at 30°C with shaking. Two hundred microlitres of cultures assayed for b-galactosidase levels at wavelength 405.

5

Two dimensional gel electrophoresis:

Rehydration:

Eighteen centimeter long Immobiline DryStrips pH 3-11 from Pharmacia were rehydrated overnight in the rehydration solution (Table 2)

10

E. coli protein sample preparation:

A two hundred milliliters of the  $\lambda$ RTL culture containing plasmids pPep(MGFWR) and TrpRep(T44M) was grown on YEA media with 25  $\mu$ g/ml chloramphenicol until an OD of 0.6 at wavelength 600nm. Likewise, two hundred milliliters of the control  $\lambda$ RTL culture containing plasmids pBAD33 and TrpT44Mrep was grown on YEA media with, 25 $\mu$ g/ml chloramphenicol until an OD of 0.6 at wavelength 600nm.

15

The two 200ml cultures were harvested and centrifuged at 5000 rpm for 10 minutes in a SS34 rotor at 4°C. Cells were re-suspended in 2ml of sample buffer I (Table 3), 40 $\mu$ l of sample buffer II (Table 2) was added and the cell paste incubated at 37°C for 60 minutes. The lysate was not

20

overly viscous at this point. Five microliters of the cell paste was added to 175  $\mu$ l of sample application buffer (Table 3). The samples were spun in a microcentrifuge for 15 minutes at maximum speed before application into the sample applicator cups of the first

25

dimension of the Pharmacia 2Dgel system.

30

Running the 2D gel:

The IPG strips were run at 150V, 1mA, 1W, for 90 minutes followed by a second step of 3000V, 1mA, 1W for 15 hours.

The strips were rinsed briefly with dH<sub>2</sub>O and equilibrated twice in 5ml ESS buffer (Table 3). Following equilibration the strips were placed on top of 8-16% gradient slab gels and encased in warm 1x cathode buffer (Table 3) with 0.6% agarose. The gradient gels were cast using a gravity driven gradient mixer with heavy solution (Table 3) in the reservoir chamber and the light solution in the mixing chamber (Table 3). To initiate polymerization at the upper light edge 300ul 15% APS was added to the light (8%) solution and 220ul 15% APS to the heavy (16%) solution.

#### Silver Staining:

Gels were silver stained as follows.

1x30 minutes in 50% methanol, %5 acetic acid.  
1x10 minutes in 50% methanol  
1x10 minutes in dH<sub>2</sub>O water  
1x1 minute in 0.02% Sodium-thiosulfate  
3x2 minutes in dH<sub>2</sub>O

1x30 minutes in cold (4°C) 0.1% silver nitrate  
3x2 minutes in dH<sub>2</sub>O  
1x1-10 minutes in 0.04% formaldehyde, 2% Sodium Carbonate with shaking

1x5 minutes in 5% acetic acid

Store gels in 1% acetic acid at 4°C protected from light.

#### Gel Analysis:

Gels were then scanned directly into the Z3 2Dgel analyses program using an UMAX MirageII scanner in transmissive mode. After analysis spots of interest were excised from the analytical gel, lyophilized, and sent for mass spectrometric identification.

#### Northern Analysis

Preparation of RNA

*E. coli*  $\lambda$ RTL<sup>F</sup> cells containing pTrpRep(T44M) and pPep(MGFLR) were grown in BTTRA and BTTRG media with, or without tryptophan, at 30°C with shaking until OD 0.6 wavelength 600 was reached. A second *E. coli*  $\lambda$ RTL<sup>F</sup> culture containing pTrpRep(WT) was grown in BTTRG culture at 30°C with shaking until OD 0.6 wavelength 600 was reached.

Five ml of boiling lysis solution (Table 3) in a 50ml conical tube (Falcon/Corning), was placed in a 100°C water bath and incubated for 10 minutes. Ten ml of each culture was mixed with the boiling lysis solution and incubated at 100°C for 5 minutes with periodic mixing. Ten ml of hot acid (65°C) phenol/chloroform was added to each tube and the tubes sealed by adding parafin oil to prevent phenol evaporation. Samples were mixed well by vortexing at high speed. Samples were further incubated for 10 minutes at 65°C with periodic mixing. Samples were spun for 15 min at 2500g (approximately 3000rpm in a Sorval RC3B rotor). The aqueous phase was carefully transferred (care was taken to avoid any of the white interface) to fresh 50ml tube and an equal volume acid phenol/chloroform was added. The tube was sealed and mixed thoroughly by vortexing at high speed. Samples were then spun 3000 rpm 15 min. (~2500g). Again the aqueous phase was transferred to a fresh 50ml conical and an equal volume of chloroform/isoamyl alcohol was added. The tube was sealed and mixed well by vortexing. After spinning for 15 minutes at 3000 rpm, the aqueous phase was transferred to new 30ml corex tube. Two volumes of 100% ethanol were added and 5M sodium acetate was added to a final concentration of 0.3M. Samples were stored at -20°C overnight. Samples were then spun for 20 minutes at 15-20,000g (12,500 rpm in SS34 rotor). The supernatant was carefully poured off and the pellet washed with 5ml of ice cold 70% EtOH (prepared with DEPC- H<sub>2</sub>O) and re-centrifuged as above. The pellet was dried at room temp for 20 minutes and resuspended in TE buffer made

with DEPC treated H<sub>2</sub>O.

DNA Hybridization Probe:

*E. coli* genomic DNA was extracted using a standard  
5 procedures and used as a template for the following PCR  
reactions. Primers pairs Amp forward, reverse, LacZ  
forward, reverse, and PheS forward, reverse were used in  
the following PCR reaction. PCR reaction: forward and  
reverse primers 20mm, template 500ng, MgCl<sub>2</sub> 3mM, dNTP 0.2  
10 mM, 1 unit pfu turbo, 5μl 10 x reaction buffer, and dH<sub>2</sub>O  
to total 50μl.

The lacZ PCR product was restricted with HindIII and  
filled with T4-DNA polymerase within the same reaction as  
suggested by the manufacturers instructions.

15 Radioactively labeled α-P32 dCTP was substituted for dCTP  
in the fill in reaction. Likewise the pheS PCR product  
was restricted with PstI and T4 filled substituting α-P32  
radioactive dCTP for dCTP. Finally the Amp PCR product  
was restricted with enzyme BanI and T4 filled  
20 substituting radioactive α-P32 dCTP for dCTP. All cut and  
labeled PCR products were gel purified using Qia quick  
PCR clean up kit.

Hybridization and Analysis

25 RNA was electrophoresed through a 1% formaldehyde gel  
according to Sambrooke et al's Manual (1989). The gel was  
capillary blotted onto nitrocellulose filter paper using  
the standard protocols. RNA was UV crosslinked by 45  
seconds of UV exposure. The filter was prehybridised for  
30 3 hours at 65°C and then hybridised for 36 hours at 65°C.  
Hybridisation buffers were in accordance with Sambrook.  
The filter was dried at room temperature and placed in a  
phosphoimager cassette overnight. The filter was then  
scanned by Molecular Dynamics phosphoimager 400e (880 E.  
35 Arques Ave, Sunnyvale CA 94086).

Results and Discussion:

An alanine to glycine substitution at position 294 (A294G) of the phenylalanyl-tRNA synthase (PheS) gene relaxes the specificity of the enzyme allowing the binding of DL-p-Cl-phenylalanine. The subsequent incorporation of DL-p-Cl-phenylalanine into the nascent peptide chain interferes with polypeptide folding and elongation resulting in cell death, a demonstration of an effective positive selection system.

Since we have placed the PheS gene under the transcriptional control of TrpR, the binding of TrpR wildtype, or a restored TrpR mutant, to the *TrpEDCBA* operator rendered the cell immune to DL-p-Cl-phenylalanine by blocking transcription of PheS gene (Table 4). Figure 10 shows the dual selection system with 2A and 2B showing PheS based selection. Colonies that falsely passed selection (Table 4) did not survive re-plating on fresh selective media.

The second reporter where the  $\beta$ -galactosidase (LacZ) gene was placed under the transcriptional control of *TrpEDCBA* operator promoter proved to be an invaluable confirmatory reporter. The LacZ gene cleaves the glycosidic linkage in X-gal to eventually produce the halogenated insoluble blue compound indigo in cells expressing LacZ. The *E. coli* cell line  $\lambda$ RTLTF has the *TrpEDCBA* operator-promoter placed in front of the lacZ gene to regulate expression but tryptophan synthesis remains unaffected. Binding of TrpR, or restored TrpR mutant to the *TrpEDCBA* operator prevents transcription of LacZ and results in a white colony phenotype. However, many colonies surviving PheS selection were blue in color, suggesting false restoration of the TrpR(T44M) mutant, and were eliminated as potential restoring peptides. The initial selection (Table 5) showed 150 surviving colonies of which 134 were white in color. The plasmids from these 134 colonies were isolated and plated

on to fresh selective plates. Only 18 of the original 134 colonies flourished on new selective media, the rest either died or were blue in color. Of the 18 colonies that tolerated DL-p-Cl-phenylalanine in the media only  
5 three gave reliable sequencing results. The results are shown in Table 6. Peptides MGFLR, and MGFWR were deduced from the DNA sequences of the three positive sequencing results.

---

~~To determine the extent of TrpR(T44M) restoration~~  
10 and tryptophan dependence, b-galactosidase activity values were calculated in the presence of peptide-expressing plasmids. Results showed that other than the positive control pTrpRep(WT), only the arabinose induced peptide sequences MGFLR and MGFWR expressed from  
15 pPep(MGFLR) / pPep(MGFWR) plasmid restored TrpR(T44M) function. The same peptides did not restore the function of another TrpR mutant G85E.

Tri and pentameric polypeptides were synthesized from the isolated peptide sequence (Table 6). Likewise,  
20 the synthetic peptides were added to the media to determine the exact level of TrpR(T44M) restoration, through  $\beta$ -galactosidase reporter activity. The addition of 1mM amounts of peptides (FWR, FLR) significantly reduced the expression of betagalactosidase from over  
25 6,000 units to under 1000 units. Consistent with peptide expression from pPepLib the synthetic peptides failed to restore function of the G85E TrpR mutant. The longer pentameric synthetic peptides failed to restore the function of either TrpR mutant, G85E or T44M.

30 The functional restoration of TrpR(T44M) was not dependent on tryptophan in the media.  $\beta$ -Galactosidase values were unaffected by the presence or absence of tryptophan for both pPep(MGFLR) and pPep(MGFWR). Only the plasmid pTrpRep(WT) was affected by the absence of  
35 tryptophan. In this case repression was slightly relieved.

The arabinose induced expression of peptide MGFLR from plasmid pPep(MGFLR) repressed the transcription of LacZ, and PheS, both in the presence and absence of tryptophan. The same plasmid could not repress  
5 transcription of LacZ or PheS in the presence of glucose. The control band  $\beta$ -lactamase remained constant and unaffected in all cases. The positive control TrpRep(WT) repressed the transcription of both LacZ and PheS.

A comparison was made of *E. coli*  $\lambda$ RTL<sup>+</sup> cells  
10 containing the TrpRep(T44M) plasmid and one of pPep(MGFWR) or pBAD33. 2DPAGE revealed a spot which is present in the pBAD33 sample but not the pPep(MGFWR) sample. Mass spectrometry identified the spot as PheS, producing 10 different peptide fragments, all of which  
15 mapped to the PheS protein.

Table 4 Verification of the reporter system

5	T44M	None	None	Tryptophan
		DL-Cl-phenyl-Alanine		
	WT	None	Positive control Colonies: ~10,000	Positive control Colonies: ~10,000
		DL-Cl-phenyl-Alanine	Negative control Colonies: 20	Negative control Colonies: 20
10		None	Positive control Colonies: ~10,000	Positive control Colonies: ~10,000
		DL-Cl-phenyl-Alanine	Negative control Colonies: 4-500	Experimental Colonies: 5,000-6,000

Table 5 Selection of Restoring Peptide:

15			pBAD33		PPepLib	
			Arabinose	Glucose	Arabinose	Glucose
20	T44M	None	Positive control Colonies: ~10,000	Positive control Colonies: ~10,000	Positive control Colonies: ~10,000	Positive control Colonies: ~10,000
		DL-Cl-phenyl-Alanine	Negative control Colonies: 20	Negative control Colonies: 20	Experimental Colonies: 150	Negative control Colonies: 20
	WT	None	Positive control Colonies: ~10,000	Positive control Colonies: ~10,000	Positive control Colonies: ~10,000	Positive control Colonies: ~10,000
		DL-Cl-phenyl-Alanine	Positive control Colonies: 5-6,000	Positive control Colonies: 5-6,000	Positive control Colonies: 5-6,000	Positive control Colonies: 5-
25						

Table 6. Sequencing results of selected pPepLib vectors

01 ~~~~~~ATGGGTTTTTGGAGGTGATAA~~~~~  
01 ~~~~~~MetGlyPheLeuArgStpStp~~~~~  
30 10 ~~~~~~ATGGGTTTTTGGAGGTGATAA~~~~~  
10 ~~~~~~MetGlyPheLeuArgStpStp~~~~~  
11 ~~~~~~ATGGGTTTTTGGAGGTGATAA~~~~~  
11 ~~~~~~MetGlyPheTrpArgStpStp~~~~~

Our experimentation strongly suggests that the function of TrpR (T44M) has been restored. Firstly the restoration is specific since the expressed peptide restored only the function of TrpR (T44M) and not that of  
5 TrpR (G85E) as judged by regulation of  $\beta$ -galactosidase reporter activity. Therefore, the peptide is not simply mimicking TrpR function, but somehow interacts specifically with TrpR (T44M) to restore function. Secondly, the regulation of two unlinked reporter systems  
10 suggests that the TrpR (T44M) is indeed binding to the *TrpEDCBA* operator fragment. Lastly, the addition of synthesized peptides identical in sequence to peptides expressed from pPepLib had an effect on reporter system regulation, suggesting that the peptide, and not the  
15 pPepLib plasmid is responsible for the regulation of reporter system expression. In no case was the wild type TrpR protein affected by the addition of peptide.

The fact that the tri-peptides (FLR, FWR) had a greater effect on the regulation of the *TrpEDCBA* linked  
20 genes *lacZ* and *pheS*, than penta-peptides may be due to increased uptake than that of the larger penta-peptides across the membrane. In support of the above argument the expression of plasmid encoded pentameric peptides did have a dramatic effect on the expression of *lacZ* and  
25 *pheS*.

The two dimensional poly acrylamide electrophoresis technique gives an *in vivo* representation of protein expression. The presence, location, and amount of reporter protein *pheS* could be determined using 2DPAGE  
30 analysis. Restoration of the TrpRT44M protein leads to reduced expression of the *TrpEDCBA* regulating *PheS*. The reduction in expressed *PheS* could be clearly seen using the 2DPAGE technique. Other than the reduction of *PheS* expression, there were few differences between control  
35 (pBAD33) protein sample and experimental (MGFLR) sample on the gels. These results argue that the function of TrpR(T44M) has indeed been restored and that the

TrpR(T44M) protein is relatively specific in its choice of the *TrpEDCBA* binding sites.

Example 7: Protocol for Generation of Peptide Expression Libraries (Microgenex Libraries)

Materials and Methods:

**1- Primers**

10      Primers: Primers used to construct Microgenex libraries.

No	Name	Sequence	bases
1	Master	AAGAGCTCGGTACCAAGAAGGAGTTTACATATGGGANNKNNKNNK TGATAAGGATCCAAGCTTGAATTCAG	71
2	Left	AAGAGCTCGGTACCAAGAAGGAG	23
15 3	Right	CTGAATTCAAGCTTGGATCCTTATC	25

The Sequencing primers used for the Microgenex verifications.

20

No	Name	Sequence	bases
1	pCEP-f	AGAGCTCGTTTAGTGAACCG	20
2	pCEP-r	GTGGTTTGTCCAAACTCATC	20
3	Left*	AAGAGCTCGGTACCAAGAAGGAG	23
25 4	Right*	CTGAATTCAAGCTTGGATCCTTATC	25

\*These primers can also be used to verify sequence of any Microgenex. Other primers within the used cloning vector can also be used.

30

**2- Cloning Vectors/ plasmids**

pCEP4 (Invitrogen): for mammalian expression.

pBAD33 (Dr. Beckwith, Harvard; Guzman et al., 1995 J. Bacteriol. 177 (14), 4121-4130) for *Escherichia coli* expression.

5     **3- *Escherichia coli* strain used for Transforming  
Constructed Microgenex libraries:**

          The Electrocompetent DH10B™ (Life Technologies, GibcoBRL) was used to transform the ligated Microgenex expression libraries.

10

**4- PCR conditions for Microgenex amplifications**

**4.1. Concentrations of primers:**

          Master primer   25 PM

          Right primer       100 PM

15           Left primer       100 PM

**4.2. PCR reactions:**

          At least two different Polymerases gave good results: Taq polymerase (Life Technologies, GibcoBRL) and rTth DNA polymerase (Perkin Elmer).

20

4.2.1.     Standard Taq polymerase as for example in SuperMix (Life Technologies, GibcoBRL).

25

          Prepare 1 to 10 PCR tubes with the following:

          90 µl SuperMix

          2 µl Master Primer (25 PM)

          4 µl Right Primer (100 PM)

1 Left Primer (100 PM)

4.2.2 The XL PCR, extra long (rTth DNA polymerase) (Perkin Elmer)

5 Also this PCR polymerase worked very well after adding buffers according to the manufacturer, but using primers as above and also the PCR cycles as indicated below.

10

4.2.3. PCR Cycles

Best Conditions were found by making a hot start at 95 for 3-5 minutes. PCR cycles were preformed only using 25 cycles. Example for PCR Cycle:

15

Hot start	95 °C	3 min.
-----------	-------	--------

25 cycles

20

Denaturation	94 °C	1 min.
--------------	-------	--------

Aneaing	38 °C	1 min.
---------	-------	--------

Elongation	60 °C	1 min.
------------	-------	--------

Hold at	4 °C	
---------	------	--

25

5. Ligation of PCR products to the corresponding expression vector:

30

PCR product was first treated with phenol-chloroform, chloroform and then precipitated with 3 volumes of cold ethanol. The DNA pellet was washed with cold 70% ethanol, dried and dissolved in appropriate volume of

H<sub>2</sub>O. DNA was then restricted by KpnI and HindIII. Restricted DNA was gel-purified and subsequently ligated to the corresponding expression vector that has been also restricted with by KpnI and HindIII. Ligation mix was precipitated and resuspended in H<sub>2</sub>O and subsequently used to transform, by electroporation, of the *Escherichia coli* strain DH10B™ (Life Technologies, GibcoBRL). Plating was done on LB ampicilin plates (200 µg/ml) for pCEP4, or chloramphenicol (40 µg/ml) for pBAD33.

#### 6. Pooling of colonies:

Colonies from all the plates were pooled together, washed with LB with ampicilin (200µg/ml) if the cloning vector contains beta-lactamase gene, such in the case of pCEP4. This step is important to get rid of cells that lack the plasmid. Pooled cell mixture was divided into small aliquots and stored at -70°C. Each aliquot contained millions of cells to ensure complexity of the Microgenex library.

#### 7. DNA Isolation of Microgenex library:

One frozen portion can be used to inoculate a one-liter culture of LB containing the appropriate antibiotic. Standard plasmid DNA isolation protocols/kits are used to isolate the DNA suitable to transform, for example mammalian cells for ligand screening.

---

Master primer was used as a template for all constructed peptide expression libraries (Microgenex) whether the expression in mammalian cells or *Escherichia coli*. It could also be used for yeast or insect cell expression. Left and Right oligos were used as forward and reverse

primers, in order to generate double-stranded DNA fragments encoding repertoire of peptide library by PCR. The first ATG in this fragment encodes Met as the translation initiation site of the peptides. Gly, the second amino acid encoding by the DNA fragment, assures the peptides expressed to be stable inside cells according to the N-end rule. The following three repeats of NNK code (N for A, C, G, and T in equal molar ratio, K for G and T in equal molar ratio) for all possible amino acids thus form the peptide

library. Two stop codons, TGA and TAA, are added right after the last NNK to stop peptide translation. The 5'-end KpnI site and 3'-end HindIII allows the fragments to be cloned into pCEP4 vector in the correct direction under CMV promoter.

The peptide-coding DNA-fragment was inserted into the mammalian expression vector pCEP4 between its KpnI and HindIII site under the CMV promoter. The plasmid has the replication origin, OriP, which enables the replication of the plasmid inside mammalian cells. Selection marker expression cassette in the plasmid provides the plasmid-transfected cells with hygromycin resistance.

After ligation of the KpnI/HindIII restricted PCR product, and the KpnI/HindIII restricted pCEP4 or pBAD 33, the DNA were electroporated into DH10B<sup>TM</sup> (Life Technologies, GibcoBRL) cells. Transformants were plated on LB plates with ampicillin (200 µg/ml) or Chloramphenicol (40 µg/ml) depending on the cloning vector used in the construction of the Microgenex library. All colonies from all the plates were pooled together, washed with LB with ampicillin (200 µg/ml), only if the cloning vector contains beta-lactamase gene, such in the case of pCEP4. Pooled cell mixture was divided into small aliquots and stored at -70°C. Each frozen portion of the Microgenex library can be used to inoculate and start a 1-liter-culture (with the appropriate antibiotic, in order to prepare the plasmid

DNA. Insertions were verified by PCR of the generated Microgenex with Right and Left oligoes. Also, the forward and reverse primers of pCEP4 were used for verifications for the constructed mammalian Microgenex. Similar PCR  
5 reactions were performed using plasmid DNA prepared from a small number of randomly-picked colonies in order to estimate the cloning efficiency and the ratio of peptide-encoding plasmids to empty vectors in any given Microgenex. Note that all generated peptides will start with **M** and **G**  
10 amino acids followed by three random amino acids (X, X, X) It should be noted that the number of the random amino acids (X) can be changed by changing the number of the codes (NNK).

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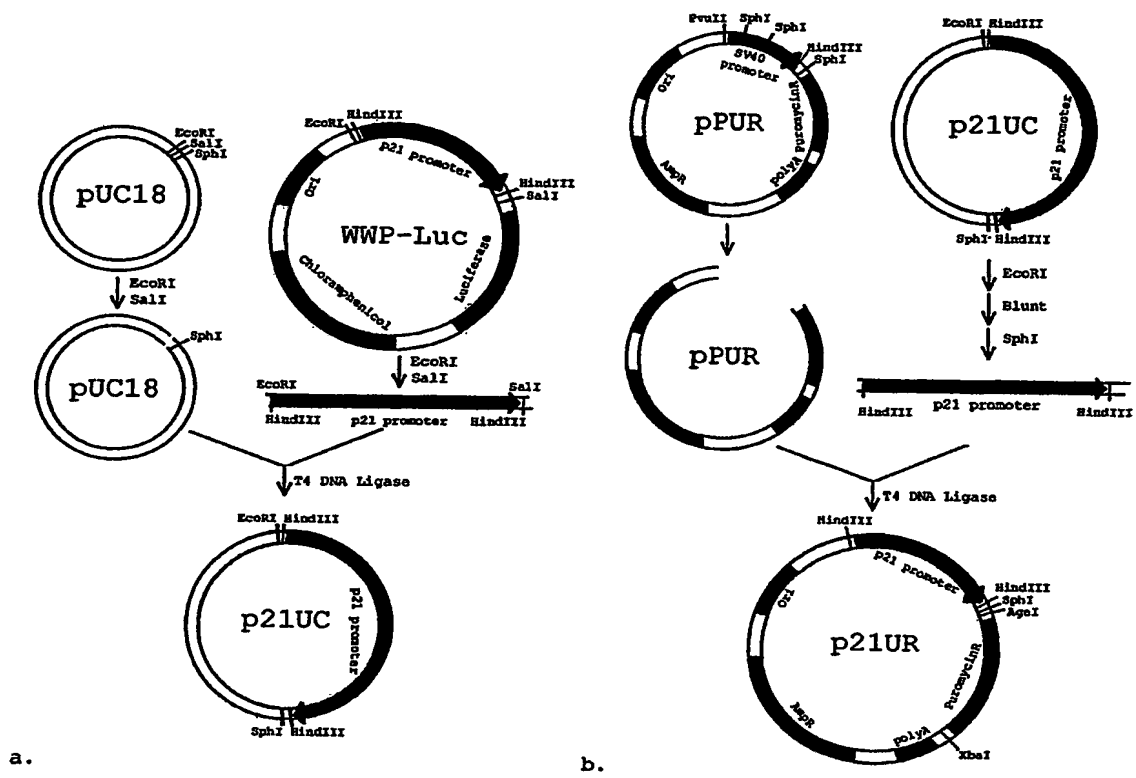


Figure 1



2/10

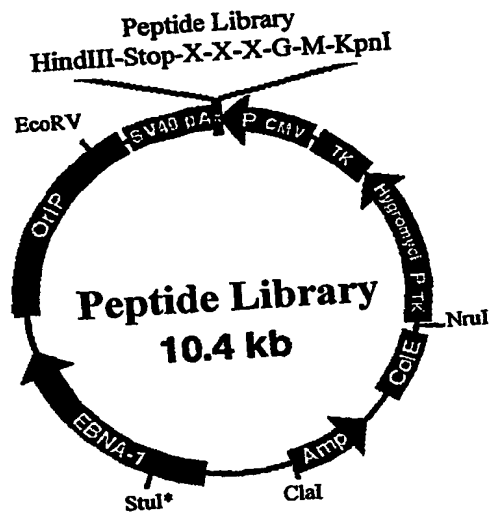
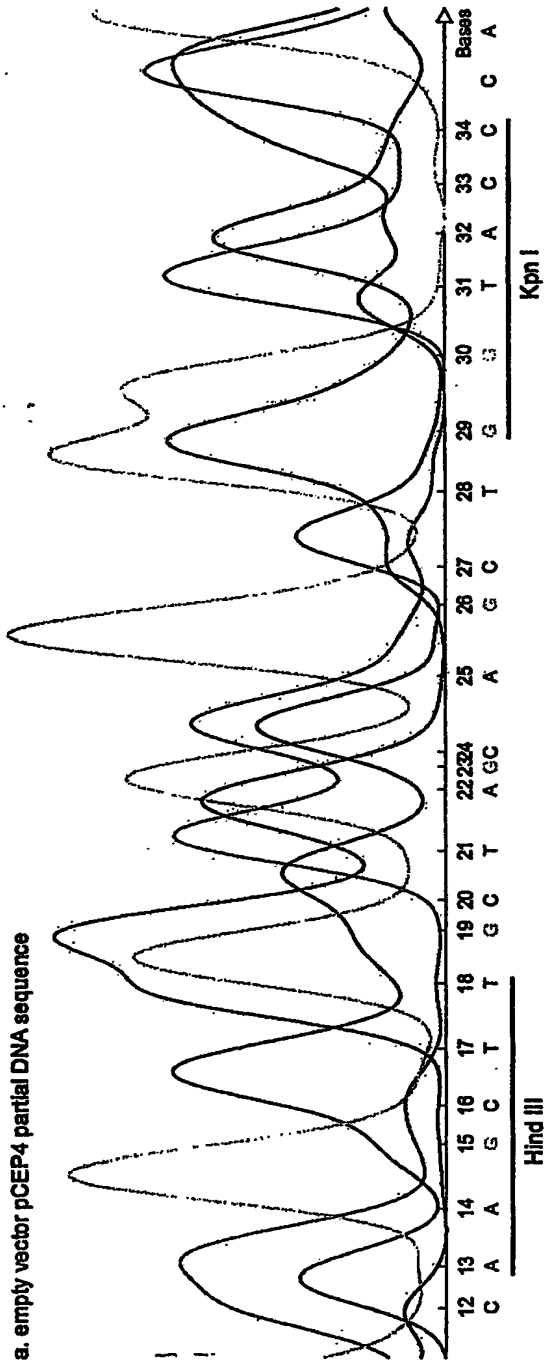


Figure 2



a. empty vector pCEP4 partial DNA sequence



b. DNA sequence of the selected ligand sitting between KpnI and Hind III of the vector pCEP4

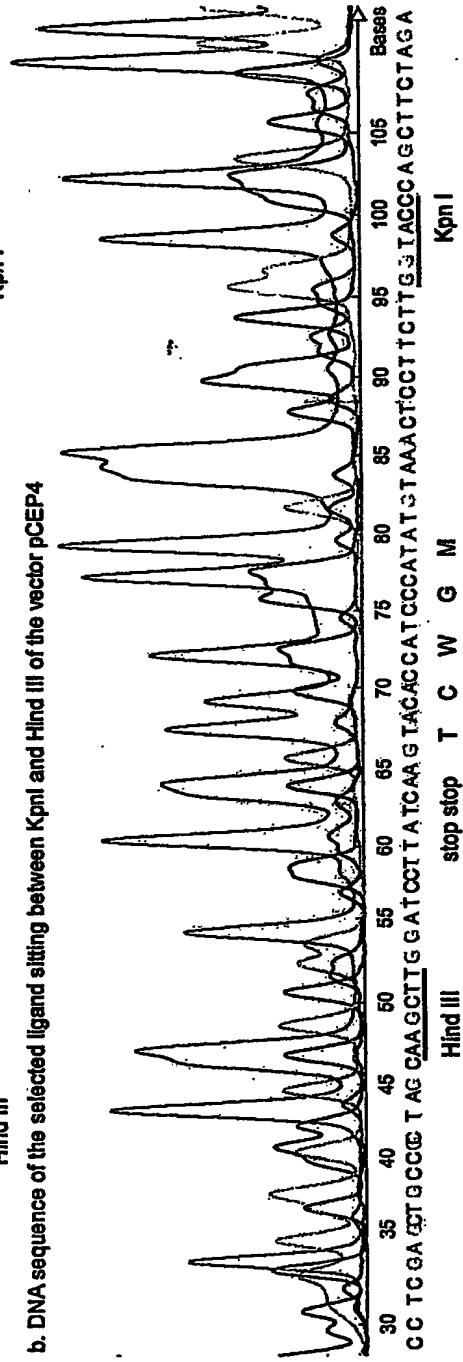


Figure 3



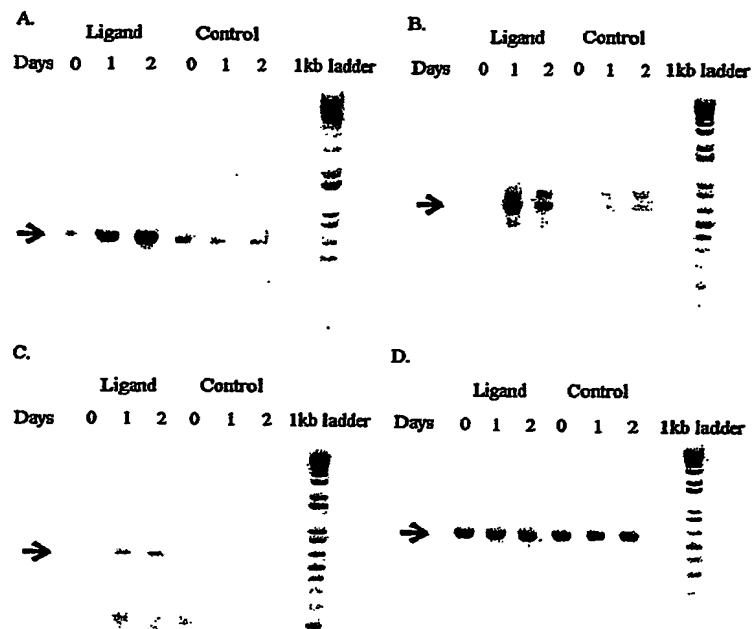
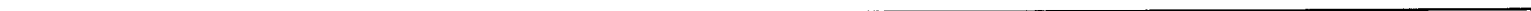


Figure 4



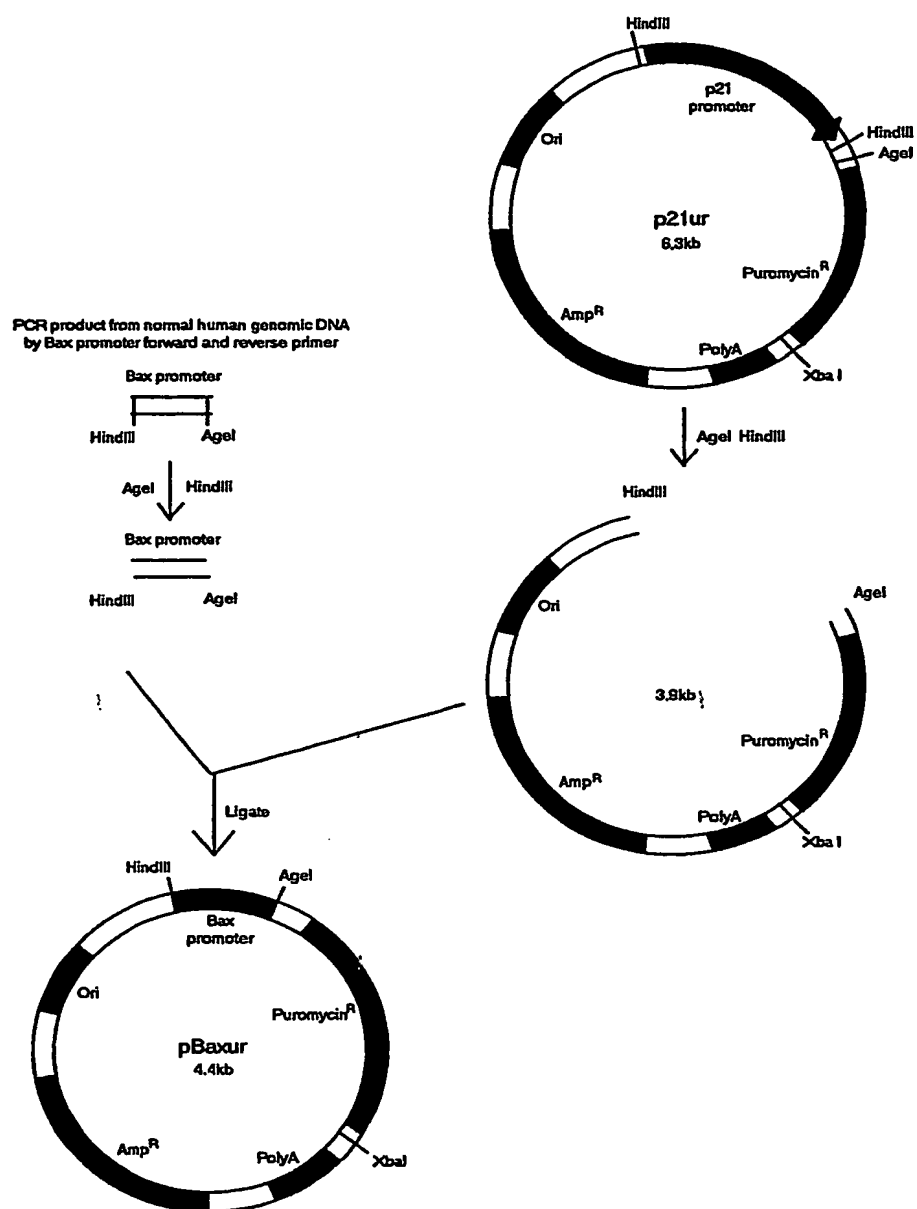


Figure 5

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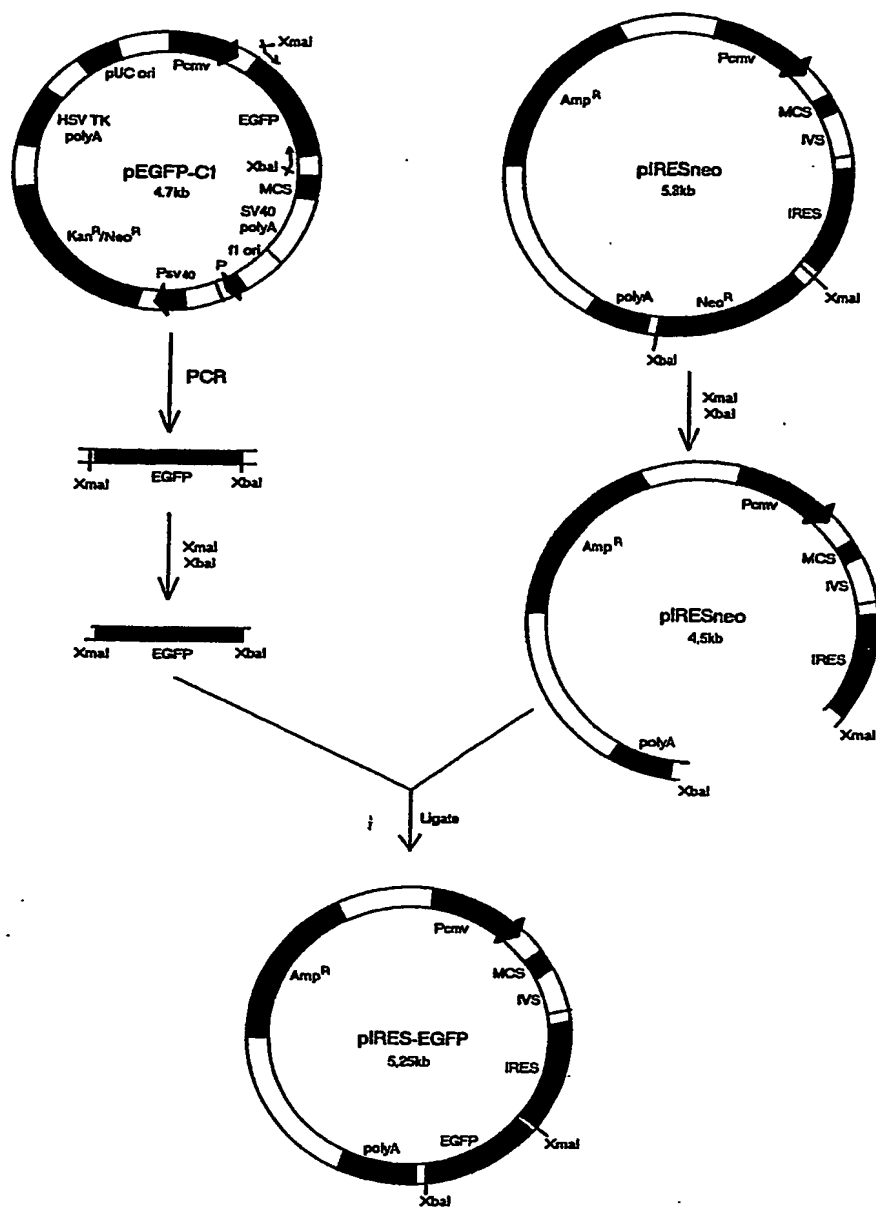


Figure 6

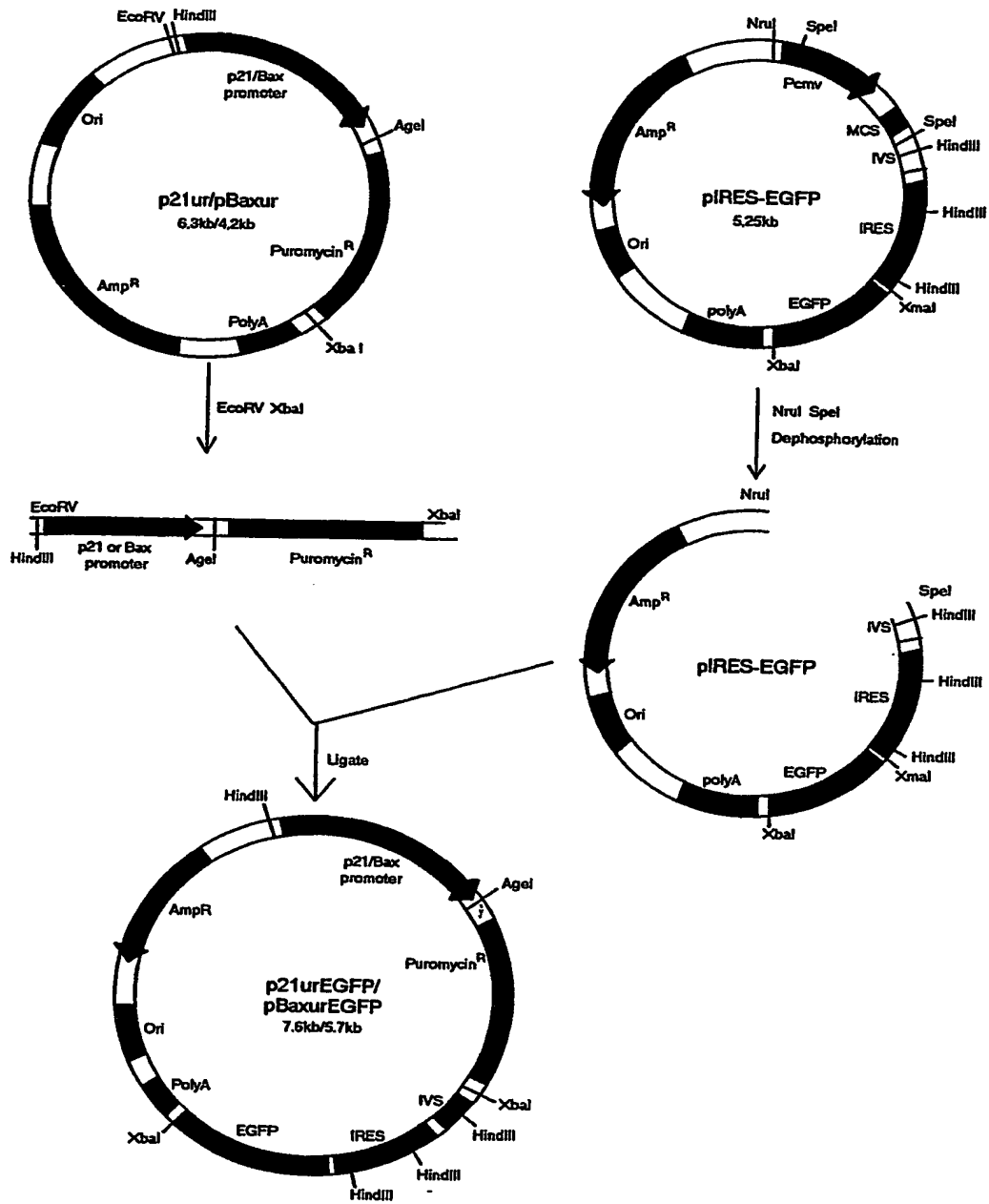


Figure 7

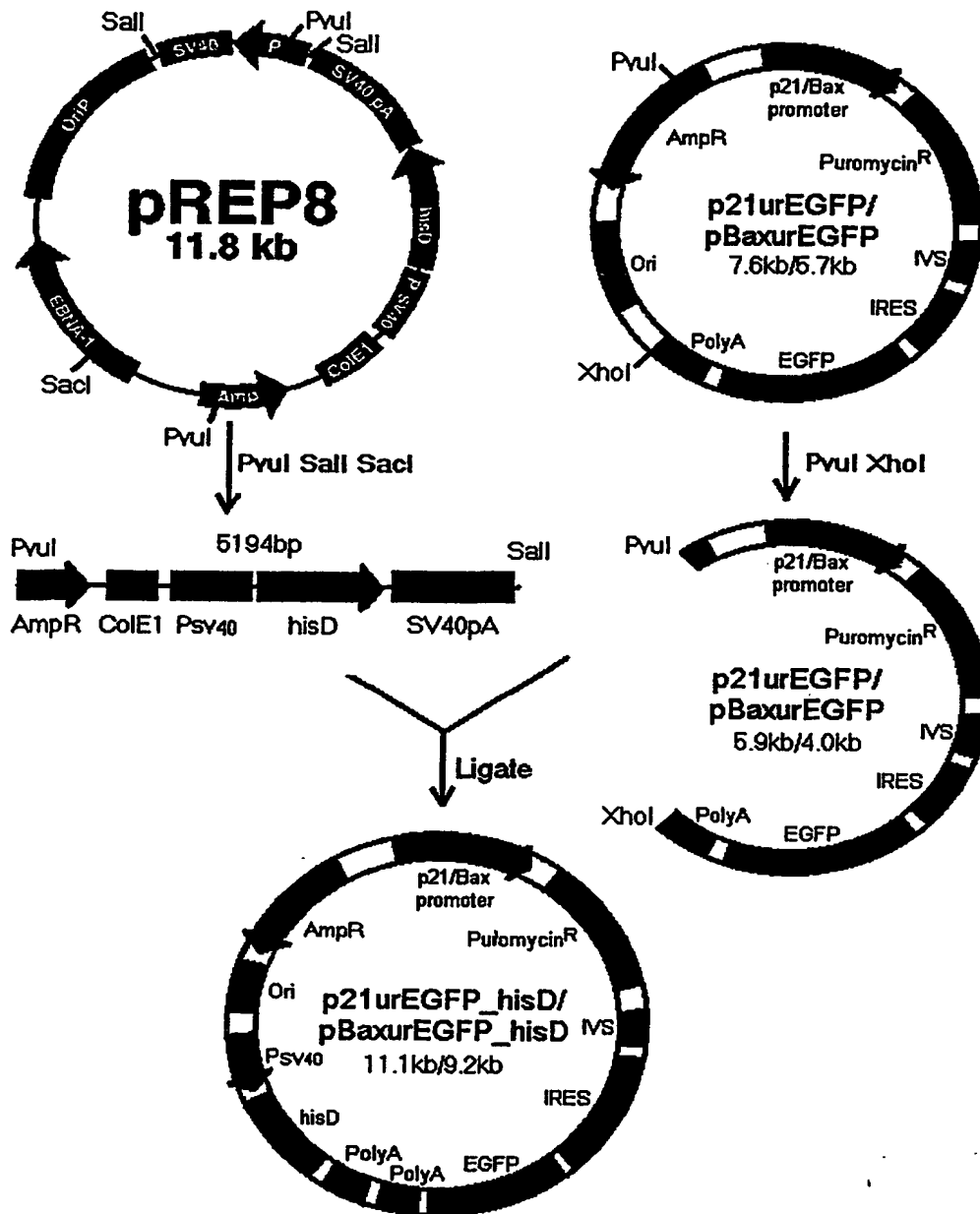


Figure 8

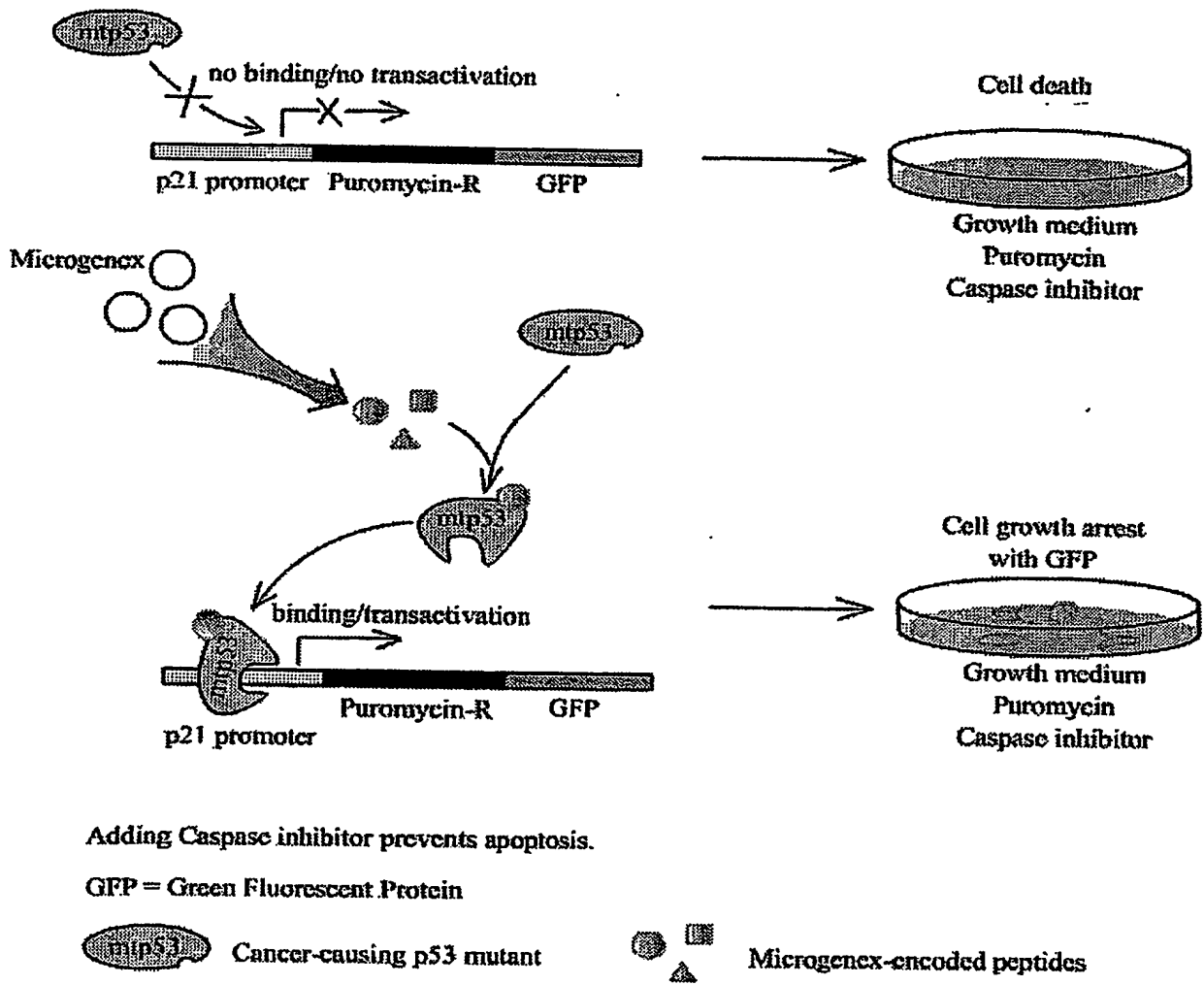


Figure 9

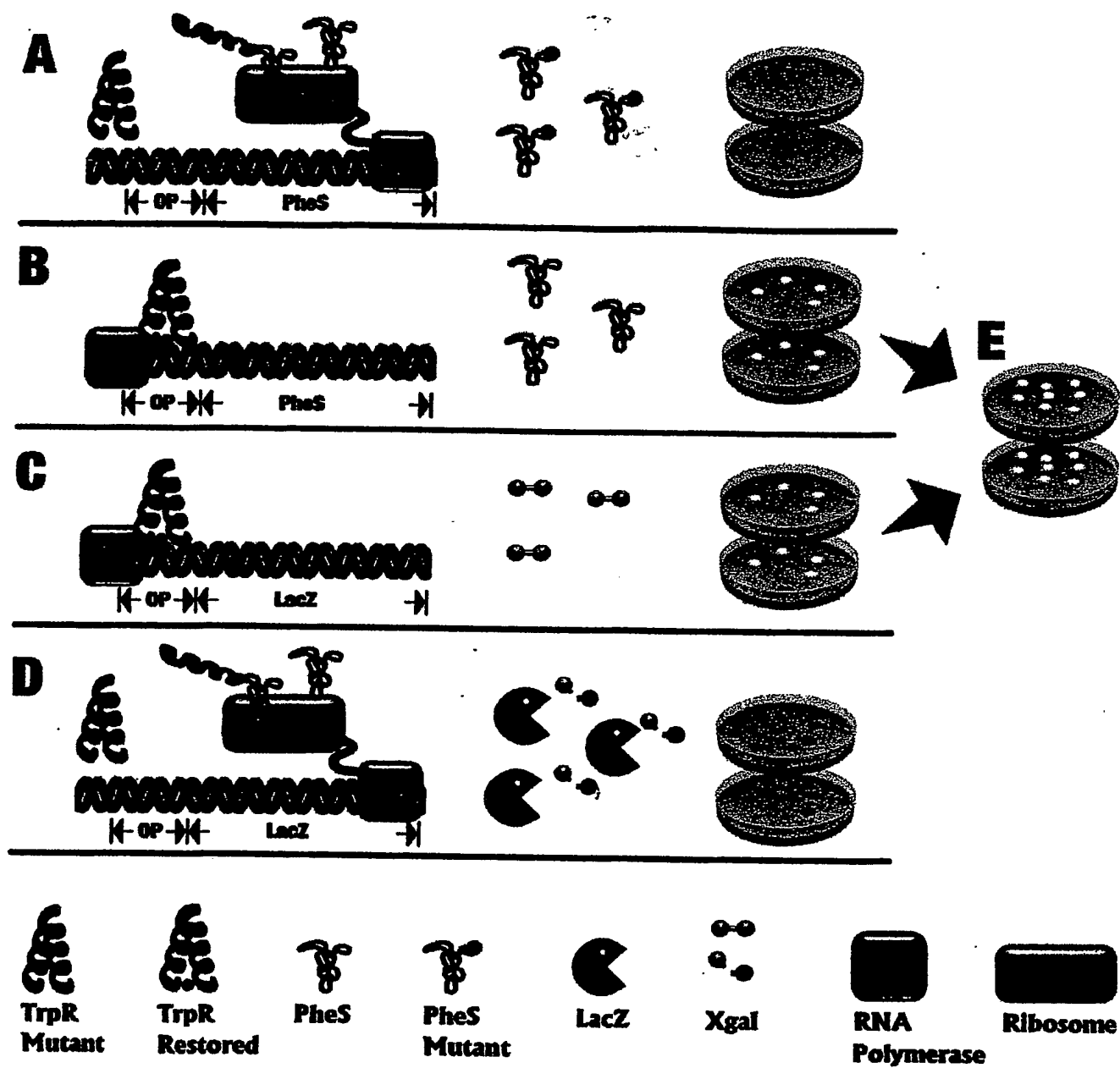


Figure 10